HISTOMORPHOLOGICAL CHARACTERIZATION OF IMMUNE SYSTEM IN GREASY GROUPER, *EPINEPHELUS TAUVINA* (FORSSKAL, 1775)

THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

Fish and Fisheries Science (Mariculture)

OF THE

CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)
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Dedicated to My Parents

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Certified that the thesis entitled **"HISTOMORPHOLOGICAL CHARACTERIZATION OF IMMUNE SYSTEM IN GREASY GROUPER, *EPINEPHELUS TAUVINA* (FORSSKAL, 1775)"** is a record of independent bonafide research work carried out by **Mr. Sushanta Kumar Patra (Ph. D. - 148)** during the period of study from September 2001 to August 2006 under our supervision and guidance for the degree of **Doctor of Philosophy** in **Fish and Fisheries Science (Mariculture)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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ACKNOWLEDGEMENTS

I am deeply indebted to Dr. K. C. George, Principal Scientist, CMFRI, Cochin for assigning me this topic. I also thank him for his invaluable advices, incessant encouragement and moral support, without which this thesis would not have seen the light of the day.

I am highly obliged to Prof (Dr.) Mohan Joseph Modayil, Director, CMFRI, Cochin, for approving my research topic and also for the infrastructural facilities provided.

I am greatly indebted to my co-guide, Sri. N.K. Sanil, Senior Scientist and In-charge , Electron Microscopy Laboratory, CMFRI, Cochin, for all the pains he had taken to help me in electron microscopy, besides his scholastic advices and erudite suggestions after critically going through the manuscript.

I place on record my indelible acknowledgements to my co-guide, Dr. K.S. Sobhana, Senior scientist (SS), CMFRI, Cochin, for the critical suggestions, timely help and encouragement throughout the period of my study at CMFRI.

I thank my advisory committee members Dr. P.C. Thomas, principal scientist, CMFRI, Cochin and Dr. T.V. Sathianandan, Senior Scientist, CMFRI, Cochin, for their invaluable advices and timely help.

I profoundly thank Dr. R. Paulraj, Principal Scientist and In-charge, Post Graduate Programme in Mariculture, CMFRI, Cochin, for the encouragement throughout the period of my study at CMFRI.

My conscience won't forgive me if I don't acknowledge the help, co-operation, moral support and encouragement rendered by my juniors and friends, Vinoj, Rajesh, Tripathy, Kali, Shine, Hina, Ravi, Pathik, Laxshmi, Surendraraj, Seema, Neetha, Thangaraj, Subodha, Behera, Chandu, Hari, Anikuttan, Anoop, Lijo, Hussain, Lijo, Reynold, Ramalinga, Joice and Nisha, I thank them all.

My special thanks are due to C. Anand, Mohan, Aruna kumar Mallick and Suresh Kamaath for the co-operation, encouragement and timely help they provided.

My special thanks to NBFRI unit, Cochin for providing me their instruments support throughout my work.

I thank Dr. J. Jayasankar, Sri. K. K. Surendran, Sri. Paulton, Sri Palaniswamy, Mrs. Vineetha, Dr (Mrs.) Gijo Ittoop, Dr(Mrs.) Imelda Joseph, Sri Chandrasekaran(PGPM), Mrs. Devaki, of PNPD and PGPM respectively for their co-operation and help.

I thank the entire staff members of Institute, library and Hindi section for helping me in collection of secondary data related to my work for the preparation of Hindi abstract.

I thank my all office staffs at K.V.K., Bhawanipatna, Kalahandi, Orissa for moral encouragement and support during entire period of my manuscript preparation.

I place on record my acknowledgments and gratitude to all my teachers whose blessings always helped me to surmount many a tussels in life.

Words cannot express my love, affection and indebtedness to my parents, brothers and sisters for their blessings, benevolence and encouragement without which I would not have reached where I am.

The financial assistance provided as senior research fellowship by the Central Institute of Fisheries Education (Deemed University, Indian Council of Agricultural Research, Versova, Mumbai) is greatly acknowledged

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31 August'2006 (Sushanta Kumar Patra)

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सारांश

ग्रीसी ग्रूपर *एपिनिफेलस टॉविना* पूरे विश्व में, लवण जल तालाबों और पंजरों में पालन की जानेवाली मछली जाति है. इस जाति की रोग प्रतिरोधता पर अधिकतर सूचना प्राप्त नहीं थी, अतः इस की रोग प्रतिरोध व्यवस्था की विशेषताओं पर जानकारी इकट्ठा करने का प्रयास किया गया. नेमी हीमटोलजिकल तकनीक, साइटोकेमिकल तरीकों और ट्रान्स्मिशन इलक्ट्रोन माइक्रोस्कोपी द्वारा इस मछली जाति के परिधीय श्वेताणुओं की विशिष्टताओं का आकलन किया गया. नेमी ऊतक विज्ञान और इलक्ट्रोन माइक्रोस्कोपी द्वारा ताइमस, वृक्क और स्प्लीन जैसे हीमोपोइटिक अंगों का अध्ययन किया गया. रक्ताणुओं और श्वेताणुओं की औसत कुल संख्या क्रमशः $4.42x10^6/m\ m^3 \pm 0.69$ और $72.75x10^3/m\ m^3 \pm 0.85$ थी. सीरम में होने वाले कुल प्रोटीन, आलबुमिन, ग्लोबुलिन तथा आलबुमिन/ग्लोबुलिन अनुपात 2.04 g % ± 0.23, 0.05 g % ± 0.01, 1.95 g % ± 0.21 और 0.02 ± 0.002 था. ग्रीसी ग्रूपर के सीरा का इलक्ट्रोफोरेटि पृथक्करण 8 से 10 पट्टियों में दिखाया पडा जिन में 4 से 5 पट्टियाँ प्रमुख थी. रक्त में मुख्यतः ग्रानुलोसाइट्स, लिम्फोसाइट्स, मोनोसाइट्स और थ्रोम्बोसाइट्स नामक चार प्रकार के श्वेताणु मौजूद थे. ग्रानुलोसाइट्स पेरोक्सिडेस, सुडान ब्लाक B, PAS के प्रति सकारात्मक और आसिड फोसफटेस के प्रति कम सकारात्मक था. ग्रानुलोसाइट्स की परासंरचना से दो प्रकार की कोशिकाएं व्यक्त हो गयी जिन में अधिकांश सजातीय और इलक्ट्रोण ज्यादा होनेवाली साइटोप्लास्मिक ग्रान्यूल थे और दूसरी कोशिकाएं क्रिस्टलोइड ग्रान्यूल लिम्फोसाइट्स की संख्या 61.83% ± 2.67 थी और मुख्यतः दो प्रकार के लिम्फोसाइट्स याने कि बडे और छोटे दिखाए पडे. लिम्फोसाइट्स की संरचना यूक्रोमाटिन और हेटरोक्रोमाटिन से युक्त केंद्रक और साइटोप्लासम में माइटोकोन्ड्रिया, असंख्य पोलीराइबोसोम्स और कच्चा एन्डोप्लास्मिक रेटिकुलम मौजूद थे. कुल ल्यूकोसाइटों में 6.33 % ± 1.30 मात्रा में मोनोसाइट्स थे और ये PAS और आसिड फोसफटेस के प्रति सकारात्मक भी थे. इनकी संरचना फेनिल साइटोप्लासम और परिधीय केंद्रक से युक्त थी. मोनोसाइटों का साइटोप्लासम लाइसोसोम, माइटोकोन्ड्रिया और कच्चा एन्डोप्लास्मिक रेटिकुलम से युक्त है. थाइमस के चारों ओर कोलाजीनस कैप्स्यूल स्थित है और कई लोब्यूल में विभाजित किया गया है. लोब्यूल रेटिकुलार कनक्टीव ऊतक से प्रबल किया गया है. इन ऊतकों में एपितीलियल कोशिकाएं और लिम्फोब्लास्ट दिखाए पडते हैं. स्प्लीन के चारों और कैप्स्यूल दिखाए पडते हैं और इन में मीसन्काइमल और पारन्काइमल ऊतकों का अनियमित वितरण देखने को मिलता है. कापिलरीस रेटिकुलार - कोलाजन फाइबर षीत से आवृत है और माक्रोफागस और लिम्फोब्लास्ट्स से भरा हुआ भी है. परासंरचना अध्ययन द्वारा स्प्लीन में रक्तोत्पत्ति की परिघटना साबित हो गयी. वृक्क के ऊतक विज्ञान और परासंरचना अध्ययनों से रक्तोत्पत्ति ऊतकों की उपस्थिति व्यक्त हो गयी. इनफ्लमेटरी अध्ययन से व्यक्त हो गया कि अंतःक्षेषण के स्थान पर न्यूट्रोफिल्स और लिम्फोब्लास्ट्स का द्वितलीय निस्राव होता है. इन्डियन इंक उपयुक्त करके किए गए जीवे फागोसाइटोसिस अध्ययन से व्यक्त हुआ कि अंतःक्षेपण के 24 घंटे बाद पेरिटोनियम में होने वाले कार्बन भरा फागोसाइट्स और फागोसाइटिक सूचक का आकलन 30.15% ± 0.46 था. PHA उपयुक्त करके किये गए त्वचा संवेदन परीक्षण से यह दिखाया पडा कि मोटापन प्रतिक्रिया 72 घंटे तक रह गयी. राबिट एरित्रोसाइट युक्त रोसेट फोर्मिंग कोशिकाएं 39.67% ± 0.71 थी.

ABSTRACT

The greasy grouper, *Epinephelus tauvina* is one of the major candidate species cultured in saltwater ponds and cages all over the world. There is scanty information on immunology of the species and thus an attempt was made to characterize the immune system of the species. The peripheral leucocytes of the species were characterized by means of routine haematological techniques, cytochemical methods and transmission electron microscopy. The haemopoietic organs like thymus, kidney and spleen of the species were studied by routine histology and electron microscopy. The mean total erythrocyte and leucocyte counts were $4.42 \times 10^{6}/mm^{3} \pm 0.69$ and $72.75 \times 10^{3}/mm^{3} \pm 0.85$ respectively. The mean serum total protein, albumin, globulin and albumin/globulin ratio were 2.04 g % ± 0.23, 0.05 g%± 0.01, 1.95 g %± 0.21 and 0.02 ± 0. 002 respectively. The electrophoretic separation of sera of the greasy grouper yielded 8 to 10 bands of which 4 to 5 bands were prominent. The differential counts of the blood contained three types of leucocytes namely granulocytes, lymphocytes and monocytes. The granulocytes were positive for peroxidase, sudan black B, PAS and mildly positive for acid phosphatase. The ultrastructure of granulocytes indicated two types of cells and the majority of them had homogeneous electron dense cytoplasmic granules while the other had crystalloid granules. The mean lymphocyte count was 61.83 % ± 2.67 and two types of lymphocytes, large and small lymphocytes, were noticed. The fine structures of the lymphocytes consisted of nucleus with euchromatin and heterochromatin and the cytoplasm contained mitochondria, numerous polyribosomes and stacks of rough endoplasmic reticulum. The monocytes formed 6.33 %± 1.30 of total leucocytes which were positive for PAS and acid phosphatase. The monocytes had foamy cytoplasm with peripherally located nucleus. The cytoplasm of monocytes contained lysosomes, mitochondria and rough endoplasmic reticulum. The thymus was surrounded by collagenous capsule and divided into several lobules by trabeculae. The lobules were supported by the reticular connective tissue, in which epithelial cells and lymphoblasts were seen. The ultrastructure of the thymus revealed capillaries lined by endothelial cells having tight junction and the lumen of the capillaries were filled with blood cells. The spleen was surrounded by the capsule and a random distribution of the mesenchymal and parenchymal tissue were noticed. The capillaries were enclosed with reticular-collagen fibre sheath and filled with macrophages and lymphoblasts. The ultrastructural studies revealed the phenomenon of haemopoieosis in spleen. The kidneys showed the presence of haemopoietic tissue in both histological and ultrastructural studies. Inflammatory studies yielded biphasic exudation of neutrophils and lymphoblasts at the site of injection. In vivo phagocytosis studies on Indian ink revealed carbon filled phagocytes in the peritoneum at 24 h post injection and the phagocytic index was 30.15% ± 0.46. The skin sensitivity test using PHA showed thickening reaction which prolonged more than 72 h. The rosette forming cells with rabbit erythrocytes were 39.67 % ± 0.71.

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INTRODUCTION

1. INTRODUCTION

Aquaculture, among the various food production sectors, happens to be one of the major sources of animal protein production. It ranks fourth in the quantity of meat produced after piggery, poultry and cattle rearing. World aquaculture production increased at an average rate of 9.2 percent per annum from 1970 to 2000 compared with only 1.4 percent per annum for capture fisheries and 2.8 percent for terrestrial farmed meat in terms of quantity. During the last three decades, aquaculture has become one of the fastest growing food producing sectors in many parts of the world. Contribution of aquaculture to global supply of fish, crustaceans, and molluscans reached about 30% (39.8 million tonnes) as reported in 2002 (FAO, 2002).

Husbandry practices have changed from extensive/semi-intensive to intensive/super-intensive systems. High population pressure per unit area has paved the way for infectious pathogens to multiply in the environment. In nature, there exists a delicate balance between the environment, host and pathogens. Altered environmental conditions along with other factors can always lead to stress in the hosts. Chronic stress may lead to disease. In fact, pathogens manipulate the delicate environment according to their requirement, which may not be suitable for the host. The change in the environment by the pathogens may be brought about by the release of biochemical molecules in the form of toxins or by change in water quality parameters or by other means. Diseases are thus recognized as one of the most important constraints in the pursuit of sustained aquaculture production (Shankar and Mohan, 2002).

In recent years, strategies aimed at preventing economically important diseases have been given a lot of attention. In the intensive system of aquaculture production, fish are cultured in high densities and the possibility of exposure to pathogens throughout the production cycle is very high. Under such conditions the problem of infectious diseases looms at large. In fact, bacterial and viral diseases of

farmed fish have led to high mortalities and reduced returns for the fish farming industry. Fish diseases particularly those caused by the bacteria are the most important causes of losses among fish farm stocks (Inglis *et al.*, 1993).

Various measures are being taken to manage diseases. Antibiotics provide a useful means for controlling many of the bacterial diseases. On the contrary they have attracted a lot of criticism also. Problems like antibiotic tissue residues in the cultured animals, bacterial drug resistance and toxicity to human beings have been documented due to the indiscriminate usage of antibiotics necessitating appropriate intervention strategies (Vinitnantharat, 2001). In this scenario, prophylaxis is becoming more and more important. In recent years, lot of attention is given to health management of aquatic animals using various immunoprophylactic techniques like vaccination and immunostimulation. For rationalization of immunoprophylactic applications, it is vital to have a thorough insight into the basic defense mechanisms of the host.

The immune system, vested with the role of defense (against foreign substances) is composed of various cell types, tissues and organs. The mechanisms of defense executed by the immune system are of non-specific and specific types. The former is encountered in almost all living organisms including fish. It is non-specific because the same immune responses can be elicited by a variety of unrelated foreign particles. Specific mechanisms of defense are found only in vertebrates and are directed against molecules that generate the stimulus. Though the defense mechanisms can be categorized into specific and non-specific groups it is important to realize that *in vivo* they act in conjunction with each other, the two systems being inter-dependent in many ways (Ellis, 2003). The sum total of defense factors responsible for immunity to a particular disease is a highly complex inter-relationship of specific and non-specific defense mechanisms. In the event of a pathogen attack, the host is capable of responding in a highly complex manner, orchestrating many of the non-specifics (both humoral and cellular) and specific defense mechanisms.

Humoral (soluble) factors of the non-specific immune system, within the body fluids, have protective functions either by inhibiting the growth of micro-organisms, or by neutralizing enzymes on which the pathogen depends. These may be classified according to their activity as: growth inhibitors (transferrin, interferon), lysins (complement, lysozymes) and precipitins and agglutinins (C-reactive protein, lectins). Non-specific cellular defense mechanisms are carried out by a variety of cells. Among them, the most important ones are the monocyte/ macrophage and the neutrophil granulocyte (Secombes, 1996).

At the center of the specific defense mechanisms is the lymphocyte, called the 'immunocompetent cell'. It is responsible for initiating and mediating the three aspects of specific immunity namely i) humoral immunity, ii) cell mediated immunity (CMI), and iii) immunological memory. Humoral immunity refers to the production of soluble antibody (immunoglobulin) while CMI refers to responses, which are mediated by a variety of cells like lymphocytes, macrophages etc. An important aspect of specific immunity is memory, which constitutes an adaptive change in the lymphoid cell populations so that on subsequent challenges by the same antigen a secondary response occurs which is characterized by a shorter latent period and enhanced magnitude.

Groupers (*Epinephelus spp.*) are highly priced and popular food fishes among the major cultured fish species in Southeast Asia (Seng *et al.*, 1998). Groupers are mainly cultured in saltwater ponds or sea cages. The greasy grouper, *Epinephelus tauvina*, is widely cultured in Southeast Asia. Its production in 1995 was about 1000 tons (Tacon, 1998). In Taiwan, *E. malabaricus, E. coioides* and *E. akaara* are widely cultured which accounts for more than 90% of all the cultured groupers. In India, the grouper, *Epinephelus tauvina* has been successfully bred and the methodology for sex inversion and brood stock development has been evolved (Devaraj *et al.*, 1999).

The grouper aquaculture industry has been severely affected by several infectious diseases. The major grouper diseases are contributed by various

pathogens, including viruses (e.g. viral nervous necrosis and iridovirus), bacteria (e.g. vibriosis: *Vibrio alginolyticus*, *V. parahaemolyticus V. anguillarum* and streptococcosis: *Streptococcus* spp.), fungus (e.g. *Ichthyophonus* spp.), parasites etc (Rasheed, 1989). Vibriosis caused by *V. anguillarum* was recorded as one of the major threat to emerging grouper aquaculture. This pathogen can cause more than 70% mortality in cultured grouper and can result in heavy economic losses (Qin *et al.*, 2003).

Understanding the immunology of a culturable species is vital for tackling problems related to diseases. Intensification can more often result in the outbreaks of several unknown diseases. In grouper culture too intensification may give rise to several diseases where the knowledge on basic immune system will be helpful in order to develop immunoprophylaxis and immunotherapy. In this context, the present study was taken up to have an understanding of the structure and function of non-specific and specific immune system in the fish, *Epinephelus tauvina*. The fish has been selected owing to the aquaculture potential and the wide distribution it enjoys. The study was conducted with the following objectives:

- To understand the histoanatomy of lymphoid organs of *Epinephelus tauvina.*
- To characterize blood leucocytes and to study the phagocytic activities.
- To study the inflammatory responses in *Epinephelus tauvina.*
- To determine total erythrocyte and leucocyte count.
- To quantify total serum protein and albumin and understand the serum protein profile.
- To understand the cell mediated immune responses.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Through studies on disease process, it is well known that a pathogen can cause disease only if it can overcome both nonspecific and specific barriers of the host and successfully establish and proliferate. Nonspecific and specific immune systems are two main components of the defense system. The former are found in all living organisms and considered non-specific because same responses can be elicited by a variety of stimuli ranging from infectious agents to inorganic irritants such as talcum powder. The immunity which an organism derives against a pathogen is the result of a delicate interaction and cooperation between the two systems (Ellis, 2003; Shankar and Mohan, 2002).

The mucus in integument (skin, gills and gut) of fish inhibits colonization of microbes by continuous sloughing. The humoral (soluble) factors of the non-specific immune system, within the body fluids, have protective functions either by inhibiting the growth of micro-organisms, or by neutralizing enzymes on which the pathogen depends. These may be classified according to their activity as: growth inhibitors (transferrin, interferon), lysins (complement, lysozymes) and precipitins and agglutinins (C-reactive protein, lectins). A variety of cells are also involved in the non-specific defense mechanisms. Two cells perform the specialised function of phagocytosis: the monocytes/ macrophage and the neutrophil granulocyte (Secombes, 1996).

Specific immune responses are found mostly in vertebrates and are specific to the antigen. It involves an adaptive change in the lymphoid system resulting in a specific immune memory. The various types of tissues and organs involved in the specific immune system include lymphoid organs like thymus, kidney, spleen, and gut associated lymphoid tissues (GALT) (Zapata, *et al.*, 1996).

2.1 FISH LEUCOCYTES

Fish leucocytes, as mammalian leucocytes, are responsible for body defense against the invaders. Leucocytes are classified into various types based on their structure, properties and functions (Ellis, 1977; Roberts and Ellis, 2003).

2.1.1 Granulocytes

Those leucocytes possessing granules are called as granulocytes. Three sub-populations of granulocytes can be distinguished in vertebrates based on their staining characteristics. Similar classification also exists in fish granulocytes (Ellis, 1977; Roberts and Ellis, 2003).

2.1.1.1 Neutrophils

The term neutrophil, or polymorphonuclear leucocyte, is drawn from human physiology. As the granules are not necessarily neutral staining, and the nucleus not being multilobed; in other species of animals, the term heterophil or, in fish, 'type I leucocyte'; have been suggested. However, in view of its wide usage the term neutrophil will be used in this description.

Neutrophils in fish are present in about the same numbers as in mammals (3-6 $\times 10^3$ /mm^3). But they comprise a much smaller proportion of the blood leucocyte population (about 6 to 8% in fish compared to 60-70% in mammals) (Ellis, 1977; Roberts and Ellis, 2003). The number of circulating neutrophils varies considerably though they comprise a smaller proportion in fishes than in mammals due to the presence of greater proportion of lymphocytes and thrombocytes in fish blood (Ellis, 1977). Neutrophils number in peripheral blood varies in different species of fishes. In brown trout it ranges from 0-15 $\times 10^3$ /mm^3 (0 to 25% of leucocytes),

while gold fish showed a wide variation from 1-2 % to 5-12% of leucocytes (Watson *et al.*, 1963; Wienreb and Wienreb, 1969; Blaxhall and Daisley, 1973).

Many fishes have only a single type of granulocyte considered as neutrophil or a cell analogous to mammalian neutrophil. Fishes like river lamprey (*Lampetra fluviatilis*), channel catfish (*Ictalurus punctatus*), South African catfish (*Clarius gariepinus*), roach (*Rutilus rutilus*), porcupine fish (*Diodon hystrix*) and plaice (*Pleuronectes platessa*) (Ellis, 1977; Radhakrishnan *et al.*, 1976; Cannon *et al.*, 1980; Hoole and Arme1982; Page and Rowley, 1983; Savage, 1983; Kusuda and Ikeda, 1987; Ainsworth and Dexiang, 1990; Ainsworth, 1992), have only neutrophil granulocytes. Temmink and Bayne (1987) have reported the existence of granulocytes that had characteristic appearance of neutrophils in carps based on ultrastructure. Hine and Wain (1988) have also characterized neutrophils of sturgeon in a similar way as in carps. Various forms of neutrophils do exist in fish. Eventhough the preferred term 'neutrophil' exists for granulocytes having neutral or translucent staining granules terms like heterophil (*Dasyatis akajei*), type I and type II (e.g. *Scyliorhinus canicula*), and L_2 (*Rutilis rutilis*) are also used to specify fish neutrophils (Morrow and Paulsford, 1980; Hoole and Arme, 1982; Parish *et al.*, 1986). Hoole and Arme (1982) revealed the phagocytic capability in the granulocytes of roach based on their ultrastructure. They considered these granulocytes as neutrophils. Type I granulocyte, a form of neutrophil, exist in dog fish (*Scyliorhinus canicula*) and these are analogous to the mammalian neutrophils (Parish *et al.*, 1986). Gardner and Yevich (1969) claimed that neutrophils were absent in cyprinodonts and considered the granulocytes present as eisonophils.

The size of neutrophils varies from 8 to 15 µm in diameter in plaice, channel catfish, *Anguilla japonica, Cyprinus carpio* and striped bass (Ellis, 1977; Breazile *et al.*, 1982; Bodammer, 1986; Kusuda and Ikeda, 1987; Temmink and Bayne, 1987).

The neutrophils of bony fish are generally round to slightly oval. However, the cell and nucleus of fish granulocytes have been found to have a variety of shapes. The cytoplasm of the neutrophils of several teleosts appear whitish to whitish-gray with an eccentric round–to–lobed nucleus when stained with Romanowsky dyes (Ellis, 1977; Cannon *et al.*, 1980; Breazile *et al.*, 1982; Ellsaesser *et al.*, 1985; Ellis & Youson, 1989; Ainsworth & Dexiang, 1990; Ainsworth, 1992). Kusuda and Ikeda (1987) described the neutrophils of *Anguilla japonica* as having an eccentric, round-to-oval bilobed nucleus and whitish-to-whitish gray cytoplasm. *Chimaera monstrosa* have irregularly shaped heterophils with a horseshoe shaped nucleus (Mattisson and Fange, 1986). The L_2 granulocytes of roach (*Rutilus rutilus*) are round and contain a lobed nucleus (Hoole and Arme, 1982). Trouts possess polymorphonuclear neutrophils (Finn and Nielson, 1971a). The nucleus of the dogfish (*Scyliorhinus canicula*) appears eccentric and polymorphic (Parish *et al.*, 1986).

Many authors studied the cytochemical characters of teleost neutrophils. Neutrophils of *Anguilla anguilla* are positive for acid phosphatase, peroxidase and esterase and negative for alkaline phosphatase (Hine *et al.*, 1986). Most circulating neutrophils of *A. australis* and *A. dieffenbachii* are negative for peroxidase activity (Hine *et al.*, 1986). Cannon *et al.* (1980) have noticed peroxidase positive granules present in channel catfish neutrophils and have attributed antibacterial and phagocytic functions. Channel catfish neutrophils are sudan black B positive and esterase negative (Ellsaesser *et al.*, 1984, 1985; Rowley *et al.*, 1988; Ainsworth, 1992; Zapata *et al.*, 1996; Moore and Hawke, 2004). The rockfish (*Sebastes schlegeli*), rainbow trout (*Oncorhynchus mykiss*) and goldfish possess peroxidase positive granules in their neutrophils (Suzuki, 1984; Temkin and McMillan, 1986). The spherical granulocytes of the turbot (*Scophthalmus maximus)* are also positive for sudanophilic granules and alkaline phosphatase (Burrows & Fletcher, 1987). Cytoplasmic glycogen clumps are found in elasmobranches (Hine and Wain, 1987). Such clumps are also evident in tilapia (*Oerochromis mossambicus)*, sea lamprey (P*etromyzan marinus*), channel catfish (*Ictalurus puntatus*) and in the goldfish (*Carassius auratus*) (Cannon *et al.*, 1980; Doggett and Harris, 1989; Ellis

and Youson, 1989; Fujimaki and Isoda, 1990; Ainsworth, 1992). Ellis (1976, 2003) has reported plaice neutrophils positive reactions while stained with periodic acid-Schiff (PAS), Sudan black B, benzidine-peroxidase, and acid and alkaline phosphatase.

The morphology, structure, staining characters and the chemical nature of granules have been used to identify the granulocytes. Large round or elongated granules are present in the cytoplasm of plaice neutrophils (Ferguson, 1975, 1976) while oval granules are present in carp (*Cyprinus carpio*) neutrophils (Cenini, 1984). Many morphological heterogeneous granules are visible in the cytoplasm of the adult river lamprey neutrophils (Page and Rowley, 1983). The heterogeneity of these granules is probably related to cell maturity rather than to subpopulations of the cells (Page and Rowley, 1983). The granules of sting ray heterophils are elongated and electron dense, with homogeneous or fibrillar content (Chiba *et al.*, 1988). The second granulocytes type of *Ginglymostoma cirratum* (probably neutrophils) has ellipsoid or spheroid granules with internal fibrillar material (Hyder *et al.*, 1983). Type II granulocytes of dogfish, analogus to neutrophils, have membrane granules smaller than those of the eosinophils (Morrow and Pulsford, 1980). Granulocytic granules appear acidophilic in channel catfish (*Ictalurus puntatus*) neutrophils and orange in cichlid (*Oreochromis mossambicus*) type II granulocytes (eosinophils) (Cannon *et al.*, 1980; Doggett and Harris, 1989). Electron micrographs of neutrophils show prominent round or oval cytoplasmic granules. Three types of granules are seen in goldfish neutrophils, with Type I being the largest and most predominant type (Fujimaki and Isoda, 1990). They vary considerably in size among vertebrates. Ultrastructurally, granules may appear as rhomboid, crystalloid to fibrous inclusions (Ferguson, 1975; Fange and Mattisson, 1981).

There is some doubt about phagocytic capacity of fish neutrophils. Neutrophils are believed to function as phagocytes at the site of inflammation (Finco-Kent and Thune, 1987; Ainsworth, 1992; Secombes, 1996; Ellis, 2003). Plaice

neutrophils failed to ingest carbon particles (Ellis, 1976). In Rainbow trout also neutrophils at the inflammatory sites failed to ingest bacteria (Klontz, 1972).

2.1.1.2 Eosinophils

Eosinophils of fish have been designated 'eosinophil leucocytes' and 'eosinophilic granulocytes'. Cells designated as eosinophilic granulocytes are mostly found in tissues. Such cells are not found in peripheral blood. Eosinophil leucocytes have been reported in the peripheral blood of primitive bony fish (Jordan, 1938; Fange, 1968; Ward, 1969). However their presence in peripheral blood of teleost is controversial. Reports of eosinophil leucocytes are rare in fish blood (Mesnil, 1895; Roberts and Ellis, 2003) and only Meinertz (1902) observed such cells in roach. However reports of eosinophilic granulocytes for a large number of species do exist (Drzewina, 1911; Kelenyi and Nemeth, 1969). Besides above, several descriptions for eosinophils like coarse eosinophilic granulocyte, fine eosinophilic granulocyte (Fange,1987), type II (*Chaenocephalus aceratus* and *Oreochromis mossambicus*) (Doggett *et al.*, 1987; Doggett and Harris,1989), type I (e.g. *Scyliorhinus canicula*) (Morrow and Pulsford,1980), type 2 (e.g. *Scyliorhinus canicula*) (Parish et al., 1986) and G1, G2, G3 and G4 for various elasmobrachs (Fange,1987; Mainwaring and Rowley,1985; Hine and Wain,1987) have also been given. Drury (1915) claimed that freshwater and marine teleost have eosinophils, which however, are present as two categories: one with stable granules and other with distorted granules. The presence of eosinophils was reported in the blood of *Salmo salar* (Conroy, 1972) and the King salmon, *Oncorhynchus tshawytscha* (Greene, 1912). Many workers strongly argued about the absence of eosinophils in the blood of brown trout and rainbow trout (Yuki, 1957; Klontz, 1972; Blaxhall and Daisley, 1973). However cells resembling eosinophils were observed in the kidney of rainbow trout (Chiller *et al.*, 1969). Ellis (1977) also has observed eosinophils in the peripheral blood of rainbow trout. Several workers observed eosinophils in the blood of goldfish and carp (Cyprinus *carpio*) and their percentage varied from 2 to 8 % of total leucocytes (Watson *et al.*, 1963; Kelenyi and Nemeth, 1969). Hines and Yashouv (1970) reported the absence

of such cells from the blood of Israeli strain of carp (Cyprinus *carpio*). Gardner and Yevich (1969) noticed only one type of granulocytes in the circulating blood of *Fundulus heteroclitus, F. majalis* and *Cyprinodon variegatus*, and all these granulocytes were eosinophils.

Eosinophils in haemopoietic tissues have been reported from the kidney of the paddle fish, *Polyodon spathula* (Downey, 1909; Clawson *et al.*, 1966), in the meninges and other haemopoietic areas of the skull in *Amia* & *Lepisosteus* spp. (Scharrer, 1944), and *Chimaera* (Fange, 1968), in the kidney of goldfish (Weinreb, 1963), rainbow trout (Chiller *et al.*, 1966) and in carp, river bleak, tench (Kelenyi and Nemeth, 1969; Smith *et al.*, 1970), and in the liver, kidney and organ of Leydig of elasmobranches (Fange,1968).

The eosinophilic granulocytes of *Chimaera monstrosa* (a holocephalan fish) preorbital tissue are roughly 15µm in diameter (Mattisson and Fange, 1986). Such cells have non-segmented, eccentric nucleus (Mattisson and Fange, 1986). In *Ginglymostoma cirratum*, the nucleus of the eosinophil is bilobed and pseudopodia usually extend from the periphery of the cell. The nucleus of the dogfish, *Scyliorhinus canicula*, type 2 granulocytes is eccentric, irregularly shaped, while type 3 granulocytes have dumb bell-shaped nucleus (Parish *et al.*, 1986). Eosinophilic granulocytes of Rajiformes are polymorphonuclear (Hine and Wain, 1987). The eosinophils isolated from peritoneal fluids of stripped bass (*Morone saxatilis*) are 9 - 15µm (Bodammer, 1986). He stated that stripped bass possessed eosinophils with oval shape and an eccentric nucleus. The cytoplasm granules were large and stained bright red with Romanowsky dye. Kelenyi and Nemeth (1969) identified eosinophil in the blood and kidney of carp, river bleak and tench. The cytoplasm contained eosinophilic granules. These granules were peroxidase positive at pH 8.0 and weakly positive at pH 12. In electron microscope granules were 0.8 to 1.4µm in diameter and in a proportion of cells crystalloid were observed. The eosinophil granules of the goldfish have a diameter of 0.4µm and granule containing dense material enclosing rod or disc-shaped inclusions (crystalloids) (Weinreb, 1963). In kidney of paddle fish

(*Blyodon spathula*) and spleen of rainbow trout, eosinophils are observed but crystalloids and granules are not reported (Clawson *et al.*, 1966; Chiller *et al.*, 1969).

Fish eosinophils have been implicated in inflammation (Drury, 1915; Bullock, 1963; Chaicharn and Bullock, 1967; Roberts and Ellis, 2003) and phagocytosis of bacteria by eosinophils in goldfish and guppies (Watson *et al.*, 1963; Jakowska and Nigrelli, 1953; Roberts and Ellis, 2003) has been reported, while phagocytosis of thorotrast and carbon particles has also been claimed (Mackmull and Michels, 1932; Weinreb and Weinreb, 1969; Roberts and Ellis, 2003) and denied (Duthie, 1939; Roberts and Ellis, 2003). The only criterion for identifying fish eosinophils is the presence of large eosinophilic granules. Fish eosinophils have phagocytic capacity and this is noticed in eosinophils of gold fish and guppies (Roberts and Ellis, 2003).

2.1.1.3 Basophils and mast cells

The presence of basophils in fish is controversial (Ellis, 1977). Many authors have reported the presence of basophilic granulocytes in the peripheral blood (Drzewina, 1911; Duthie, 1939; Catton, 1951; Watson *et al.*, 1956; Ostroumova, 1960; Watson *et al.*, 1963; Weinreb, 1963; Lukina, 1965; Haider, 1968; Ward, 1969). Several workers were unable to find basophils in fish (Drzewina, 1911; Jordan, 1938; Duthie, 1939; Catton, 1951; Yokoyama, 1960; Klontz, 1972; Blaxhall and Daisley, 1973; Ellis, 1976).

Drzewina (1911) reported about the rare presence of orthobasophilic granulocytes in a few species out of the total 68 species studied. Catton (1951) and Duthie (1939) found them in *Triglida* spp too. Among the species of salmons, Ostroumova (1960) reported such cells from pink salmon, *Oncorhynchus gorbuscha*, Lukina (1965) in alevins of chum salmon, *Oncorhynchus keta,* and Watson *et al.* (1956) also found such cells in fingerlings of sock eye salmon, *Oncorhynchus nerka*. Watson *et al.* (1963) and Weinreb (1963) described basophils stained with

Romanowsky dyes in the goldfish. The cell had a large eccentric nucleus with homogeneous chromatin and basophilic cytoplasmic granules stained purplish blue. The granules are 0.8 µm in diameter and lacked any internal structure even in the electron microscopy. Watson *et al.* (1963) stated that basophils were fragile and destroyed during smear preparation. Michels (1923) demonstrated metachromasia on staining with thionine for granules of basophils in *Leuciscus leuciscus* and *Cyprinus carpio.* Duthie (1939) reported that basophils of *Triglidae* spp. were peroxidase negative. Pitombeira & Martins (1970) showed granules in Spanish mackerel basophils. These granules were PAS positive. They form about 1% of the circulating leucocytes in Australian lungfish, *Neoceratodus forsteri* (Ward, 1969).

Basophils are not present in the blood of eel *Anguilla* spp. (Drzewina, 1911; Michels, 1923). Yokoyama (1960) claimed them to be absent from the blood of perch. Ellis (1976) could not observe them in the blood of plaice, *Pleuronectes platessa.* Catton (1951) and Duthie (1939) also could not observe basophil granulocytes in *Ctenolabrus* spp. They are reported to be absent from the blood of brown trout (Blaxhall and Daisley, 1973) and rainbow trout (Klontz, 1972). Such cells too are absent in the blood of cyclostomes (Jordan, 1938).

The functions of basophils are not clear though they contain 5-hydroxytryptamine (5HT). The cells have not been attributed with any defense role in the fish (Roberts and Ellis, 2003).

2.1.1.3.1 Mast cells

Mast cells are present in connective tissue and found very close to small blood vessels. They originate from undifferentiated mesenchymatous cells and also from the division of already existing mast cells. They contain metachromatic granules and these granules are the source of vaso-active amines like histamine, serotonin etc. They are very common in mammals. They are also present in fishes (Robert *et al.*, 1971, 1973; Bullock *et al.*, 1976). Bucke (1972) reported mast cells in

goldfish. Anaphylaxis, causing the contraction of smooth muscle, dilation of blood vessels and increased vascular permeability is a very common reaction in mammals but could not be demonstrated in fishes (Dreyer and King, 1948; Lukyanenko, 1967; Hodgins *et al.*, 1967; Clem and Leslie, 1969; Harris, 1973). On the other hand Fletcher and Baldo (1974) reported anaphylaxis in plaice. Clearly, the functional significance of mast cells in fishes still remains to be understood.

Some fishes possess eosinophilic granular cells inside connective tissue and intestinal walls. They resemble the mast cells but stain differently (Reite, 1998). In many respect they behave like mast cells of mammals. They lack histamine but possess serotonin. Substance like C-polysaccharide causes degranulation in these cells.

2.1. 2 Lymphocytes

The vertebrate lymphocytes are identified by their cellular morphology. The lymphocytes are highly differentiated cells and are responsible for immunological reactions (White, 1963).

The morphology of lymphocytes is similar in all vertebrates, though there are minor variations in size. Two types of lymphocytes can be differentiated based on their size: one is large and the other is small. These two types do not form separate populations but represent different functions.

Watson *et al.* (1963) and Weinreb and Weinreb (1969) reported in goldfish lymphocytes comprise about 30 % and 71-82 % of all blood leucocytes respectively. In the same study, Watson *et al.* claimed 70 % of all leucocytes as thrombocytes while Weinreb and Weinreb's figure placed that as 3-13%. Similarly, the ratio of lymphocytes to thrombocytes for the blood of rainbow trout *Salmo gairdneri* was given by McCarthy *et al.* (1973) as 25:1 while Weinreb (1958) claimed

a ratio of 2:1. Catton (1951) could not detect any thrombocytes in the blood of perch. Rapidity of fish blood clot may result in an increase in the fragility of thrombocytes so that on preparing a blood smear the cytoplasm is stripped away leaving denuded nuclei, which may be mistaken for small lymphocytes (Ellis, 1976). Either lymphocyte or thrombocyte could be distinguished from each other by using the common histochemical and Romanowsky stains (Ellis, 1976). However, these cells may be more accurately determined by immunofluorescent techniques which stain only lymphocytes by virtue of the immunoglobulin present on their surface membrane (Ellis, 1976; Ellis and Parkhouse, 1975). By carrying out the staining procedure in the presence of heparin or alsever's solution to stabilize the thrombocytes, a differential count of lymphocytes and thrombocytes in the plaice revealed a ratio of 1:3. This represents about $12X10^3$ lymphocytes/mm^3. It is thus apparent that the fish blood contains a much higher density of lymphocytes than mammalian blood which averages $2\ X10^3$ lymphocyte/mm^3.

The number of lymphocytes in the peripheral circulation as well as presence of lymphocytes in various tissue are being modulated by physiological and pathological conditions (Watson *et al.*, 1963; Weinreb and Weinreb ,1969; Klontz, 1972 ; McCarthy *et al.* ,1973 ; Blaxhall & Daisley, 1973; Davidson *et al.*,1991; Davidson *et al.*,1993a; McMillan and Secombes, 1997; Lin *et al.*,1999).

Saunders (1968) described the morphology of thrombocytes in over 225 species of fish and found significant variation of cell size, shape, and staining characteristics of nucleus and cytoplasm among the various species. Gardner & Yevich (1969) examined the thrombocytes in three species of cyprinodonts and found seasonal variation in their numbers. The percentages of cells were: lymphocytes, 2-13% (the lowest figure was recorded in winter, the highest in summer, and the average being 7-8%); thrombocytes, 82-95%.

In the teleost, *Pleuronectes platessa*, the average diameter of small lymphocyte is 4.5 µm where as in goldfish, *Carassius auratus*, 8.2 µm (Weinreb, 1963; Ellis, 1976). Large lymphocytes may have 12-µm diameter and have large cytoplasm. The nucleus occupies the major portion of the cell with a narrow rim of basophilic cytoplasm. The cytoplasm contains mitochondria and ribosomes (Ferguson, 1976). Small lymphocytes constitute about 90% of blood lymphocytes. Majority of small lymphocytes appear as inactive undifferentiated cells. They circulated in this form till stimulation by specific antigen (Roberts and Ellis, 2003).

The fine structure of fish lymphocytes is very similar to mammalian lymphocytes. Cytoplasm has numerous mitochondria, rough and smooth endoplasmic reticulum, abundant ribosomes and Golgi vesicles (Weinreb, 1963; Ferguson, 1976).

Some lymphocytes have reddish purple azurophilic granules. They are demonstrated in fish lymphocytes by histochemical methods like Sudan black B, benzidine peroxidase and the acid and alkaline phosphatase tests (Ellis, 1976). Plaice lymphocytes possess slight Periodic Acid Schiff (PAS) positivity.

The lymphocytes are present in blood and lymph and in lymphoid organs. In most fishes, spleen and kidney are the major lymphoid organs (Davidson *et al.*, 1993 a, b, 1997; Lin *et al.*, 1998).

In mammals recirculatory pathways of lymphocytes are clearly defined whereas in fish it has not been worked out. The lymphocytes can be seen in neural lymphatic duct that receives lymph from body muscle and this lymph returned to the blood system via duct of Cuvier. These lymphocytes preferentially settled in kidney and spleen (Wardle, 1971; Klontz, 1972; Ellis and de Sousa, 1974).

2.1.3 Monocytes and Macrophages

Monocytes are part of the mononuclear phagocytic system and are not fully differentiated. They enter into peripheral blood and become mature phagocytes in various tissues. In fishes, the mononuclear phagocytic system consists of circulating monocytes, connective tissue phagocytes, macrophages of spleen and interstitial renal tissue and lining endocardial cells of cardiac atrium (Ellis, 1981; Tizard, 1992).

Monocytes of plaice form about 0.1% of the circulating leucocyte and arise from renal haematopoietic tissue (Ellis *et al.*, 1976; Roberts and Ellis, 2003). They also possess a few fine scattered granules that are positive for PAS and acid phosphatase stain. The cell membrane is thrown into pseudopodia and the nucleus is situated eccentrically with chromatin dispersed marginally. It has got a well-developed Golgi apparatus and lysosomes that vary in size (Roberts and Ellis, 2003).

Monocytes are capable of phagocytosis and they are able to ingest colloidal particles (Weinreb and Weinreb, 1969; Ellis *et al.*, 1976; Roberts and Ellis, 2003).

2.1.3.1 Macrophages

Macrophages are matured cells arising out of circulating monocytes. They are found in connective tissues and abundant in the heart, mesentery, peritoneal cavity, kidney, and spleen (Mackmull and Michels, 1932; Ellis *et al.*, 1976; Ellis, 1981; Afonso *et al.*, 1998a; Lin *et al.*, 1998; Press and Evensen, 1999). They are also found abundantly in endocardial- lining cells of atrium of heart (Ellis, 1974; Ferguson, 1975; Press and Evenson, 1999). Macrophages accumulate in lymphoid organs and other parenchymatous organ forming nodules of melano-macrophage centers. This center contains phagocytosed macrophages and melanin (Roberts, 1974; Ellis, 1981). The presence of melanin in melanomacrophage centers may be

related to releasing hydrogen peroxidase from NADH that might be employed in bactericidal process (Edelstein, 1971). They are also able to ingest soluble antigens (Ellis, 1974).

Mawdesley-Thomas and Young (1967) noticed melanin- containing cells in injured tissue of the gold fish. *Cryptocotyle metacercarie* were encysted in the presence of melanin containing cells in plaice (McQueen *et al.*, 1973). During severe septicaemia in *Salmo trutta*, melanin-containing macrophages from the kidney and spleen migrate into the circulation (Thorpe and Roberts, 1972).

The role of macrophages in antigen presentation and processing in specific immune response is not established in fish macrophages but the presence of macrophages in association with antibody producing lymphoid organs indicate they are playing significant role (Klontz, 1972).

Macrophages contain antibodies on their cell membrane surface. These immunoglobulin molecules have been demonstrated by fluorescent-labeling in plaice spleen and kidney (Ellis, 1974). Macrophages from the kidney and spleen of rainbow trout immunized against sheep erythrocytes would form rosettes which suggested that antibody was present on macrophages surface (Chiller *et al.*, 1969).

2.1. 4 Thrombocytes

There are four morphological types of thrombocytes in fish. They are spike, spindle, oval and lone nucleus forms. Oval and lone nuclear forms are difficult to differentiate from small leucocytes. The spent thrombocytes may be confused with lymphocytes. Hence the differential counts may go wrong (Roberts and Ellis, 2003). Hines and Yashouv (1970) reported that in common carp, *Cyprinus carpio* 4% of leucocytes counted might be spent thrombocytes. Ellis (1976) used fluorescent antibody technique to differentiate thrombocytes from lymphocytes and reported the

ratio of thrombocytes to lymphocytes was 1.4 to 1. The total numbers of thrombocytes in peripheral blood ranged from 60, 000 to 70, 000/mm^3. Gardner & Yevich (1969) studied the thrombocytes in three species of teleosts stained with Romanowsky dyes. Thrombocytes contain spheroid granules inside cytoplasm. These granules were either grouped at one pole or scattered through out the cytoplasm. These granules are deeply basophilic in young thrombocytes, *lightly* basophilic in intermediate forms and stained reddish pink in mature thrombocytes. The cytoplasm of immature cell appeared blue while mature cells looked grayish blue. In phase contrast microscope, a refractive vacuole could be seen at the base end of the thrombocytes. Cytoplasmic granules were not observed in thrombocytes of plaice (Ellis, 1976). However acid phosphatase positive cytoplasmic granules were observed in plaice and these granules usually appeared as single granule at the base of spiked pole. A number of coarse PAS positive granules are also observed in plaice. The peroxidase reaction was negative for thrombocytes of the fish (Yuki, 1957). Thrombocytes of brown trout *Salmo trutta* were negative for PAS, Sudan black B and benzidine-peroxidase (Blaxhall and Daisley, 1973).

In electron microscopy, the nuclear chromatin was arranged in ribbed pattern and cytoplasm contains many vesicles. A labyrinth interconnecting these vesicles ramified through cytoplasm and open through fenestrate to exterior (Ferguson, 1976).

The origin of thrombocyte is not known clearly. Probably they arise from stem cell called small lymphoid haemoblast (Catton, 1951, Klontz, 1972). Some people view lymphocytes as ancestors of the thrombocytes (Jordon, 1926; Jordan and Speidle, 1923, 1924a, b, 1930; Gardner and Yevich, 1969). Ferguson (1976) is of the view that they are originating from spleenic tissue.

2.2 LYMPHOID ORGANS

The primary source of lymphohaemopoietic tissue in higher vertebrates includes the lymph nodes (lymphoid), bone marrow (myeloid), and spleen. Teleosts have a well- developed thymus and spleen, but lack lymph nodes and bone marrow. Lymphomyeloid tissues are found in the anterior part of the teleost kidney (the head-kidney or pronephros) and perform similar functions to bone marrow and lymph nodes in mammals (Moore and Hawke, 2004).

2.2.1 Thymus

Thymus like gland was first noticed in the most primitive cyclostomes like hagfishes and lampreys. In hagfish it appears as lymphoid cell accumulation in pharyngeal velar muscle (Riviere *et al.*, 1975). In lamprey pharyngeal epithelium contains lymphocytic accumulations (Harboe, 1963; Page and Rowley, 1982; Ardavin and Zapata, 1988). This lymphoid tissue is found in the blood sinuses of pharyngeal lamina propria and considered as equivalent to the thymus (Good *et al.*, 1966; Shields *et al.*, 1979). Ardavin and Zapata (1988) based on the presence of lymphoid accumulations however denied any similarity to the thymus of highly evolved vertebrates. The first appearance of well-developed thymus gland is found in cartilaginous and bony fishes (Chilmonczyk, 1992).

The thymus originates from the primitive pharynx arising as thymic buds from the branchial pouches or pharyngeal pouches. All these primodia develop into thymus (Manning, 1981). The thymus is the first lymphoid organ in which lymphocytes appear (Ellis, 1977; Chilmonczyk, 1992). The lymphocytes infiltrate pharyngeal epithelium of pharyngeal pouch. In *Salmo salar* the pouch was infiltrated by lymphocytes 22 days pre-hatch. In *Oncorhynchus mykiss* lymphocytes appeared 5 days before hatching. Delayed infiltrations were observed in *Harpagifer antarcticus* and in channel catfish. The thymocytes appeared before the development of blood

vascular system (Grizzle and Rogers, 1976; Ellis, 1977; Grace & Manning, 1980; O'Neill, 1989).

Compared to the mammal, fish thymus occupies a much superficial position just behind the gill chamber epithelium (Grace and Manning, 1980; Zapata, 1981a,b; Chilmonczyk, 1992). The thymus is a paired organ and is situated in dorso-lateral region of the gill chamber (Chilmonczyk, 1992).

There is lot of variation among the fishes on the histomorphology of thymus. Thymus occupies superficially and covered by pharyngeal mucosa, the gland has got a connective tissue capsule that projected as several trabeculae within the parenchyma. This trabecula gives a lobulated appearance for thymus glands in many fishes, whereas number of fishes, the same is not lobulated (Lagabrielle, 1938; Good *et al.*, 1966; Sailendri and Muthukkaruppan, 1975; Ghoneum and Egami, 1982; Teshima and Tomonaga, 1986; Chilmonczyk, 1992).

Thymic lobules contain primitive lymphoid cells such as thymocytes and non-lymphoid epithelial cell. The stroma of the thymus is made of reticular tissue and this reticular framework supports the thymocytes, mesenchyma cells and epithelial cell. The epithelial cells are also having secretory role (Castillo *et al.*, 1990).

In mammal thymus lobules has got two distinct zones namely the cortex and medulla. But such boundaries are not clearly defined in fish. However for convenience it can be divided as outer, middle and inner zones. Outer zones are highly lymphoid while middle and inner zones contain more mature lymphocytes with abundant connective tissue (Iwama & Nakanishi, 1996).

Thymus in cartilaginous fish, holocephalan and elasmobranches are divided into cortex and medulla (Good *et al.*, 1966; Zapata, 1980; Pulsford *et al.*, 1984; Matisson and Fange, 1986). The structure of thymus in teleosts varies

considerably. Cortex and medulla differentiation is noticed in *Zoarces viviparus,* channel catfish (*Ictalurus punctatus)*, and grey mullet (*Chelon labrosus)* (Bly, 1985; Mughal and Manning, 1986; Ellsaesser *et al.*, 1988). Three zones are reported in *Tilapia mossambica* and rainbow trout, *Onchorhynchus mykiss* (Sailendri and Muthukkaruppan, 1975; Tatner and Manning, 1982; Chilmonczyk, 1983). The thymus of the pike, *Esox lucius,* has four zones (Mulcahy, 1970).

The major cell component of the thymus is the lymphoid cell that includes lymphocytes and lymphoblasts. Each sub-population can be characterized by ultrastructure studies (Mulcahy, 1970; Zapata, 1980; Pulsford *et al.*, 1984). Most lymphocytes of thymus appear quiescent but others are undergoing divisions. These divisions can be seen in all zones (Zapata, 1980, Botham and Manning, 1981; Tatner and Manning, 1983). In mammals and birds there is a flow of pre-thymic stem cells from haemopoietic tissue to the thymus. Once these cells enter the thymus they undergo rapid mitosis and many newly formed cells die within the thymus. The dynamic of lymphoid proliferation in thymus of fish have not been studied yet. Tatner (1985) demonstrated migration of lymphoid cells from thymus to the peripheral lymphoid organs of trout *Onchorhynchus mykiss*. The author showed heavy migration from thymus to spleen and kidney. The labeled lymphocytes settled preferentially in spleen than in kidney. In mammal only a small percentage of thymic cells become part of the peripheral T cell pool (Scolly *et al.*, 1980). Early ablation of thymus depletes the number of cell in spleen (Grace and Manning, 1980). In plaice *Pleuronectes platessa* peripheral lymphocytes rarely migrate to thymus (Ellis and de Sousa, 1974). Whereas in rainbow trout peripheral lymphocytes move through thymus and settled at spleen and kidney and there is ecotaxis of T cells (De sousa, 1971; Tatner and Findlay, 1991).

Hassall's corpuscles were formed by the epithelial cells of thymus in higher vertebrates (Kendall, 1981). Such a structure is rarely noticed in teleost fish though structure analogous to Hassall's corpuscles has been reported in some fishes. Rounded accumulations of reticular endothelial cells are also seen. This

structure in electron microscopy has been shown heterogeneous composition of epithelial cells, macrophages, polymorphonuclar cells and keratin (Blau, 1973; Sailendri and Muthukkaruppan, 1975; Ellsaesser *et al.*, 1988). The vascular structure of thymus in teleost presents some features similar to Hassall's corpuscles (Belova, 1976; Chilmonczyk, 1983; O'Neill, 1989). Secretory epithelial cells in thymus have been reported (Gorgollon, 1983; Fange and Pulsford, 1985). However epithelial cell network in thymus is present in fishes. These stromal cells are denser in the capsule and inner zone than in cortical zone.

Thymatic epithelial cells in the rainbow trout can be divided into two types based on the presence of keratin compounds (Castillo *et al.*, 1990). Seven-sub populations of the epithelial cells are also described based on the location within the gland, their morphology and histochemical activities. The epithelial reticular cells are the first cell type of non-lymphoid element to appear in the thymus. The inner zone of the thymus mainly consists of thymoblast and its derivative thymatic epithelium (Chantanachookhin *et al.*, 1991). Castillo *et al.*, 1991 stated that the epithelial cells could be classified into three types based on their ultrastructural character and location: (a) limiting epithelial cells adjacent to the capsule (b) dark epithelial stellate cells with an electron dense cytoplasm and (c) pale epithelial cells characterized by electro lucent cytoplasm, which display the secretory function. Pale and dark epithelial cells in the thymus were also reported from common sole *Solea solea* (Pulsford *et al.*, 1991). There is lack of information on blood supply to the thymus. The vascularization of the thymus is closely associated with connective tissue septa and this vascularization is derived from gill vascular system and its arterial origin. The thymus artery is a branch of segmental artery. In the cephalic part of the organ, the arteries split and form a dense network of capillaries and this empty into vein (Belova, 1976; Chilmonczyk, 1983).

Macrophages are also occasionally found in all vertebrate thymus. Accumulation of macrophages was noticed in inflamed thymus of *Salmo salar*. The origin of macrophages is not known. They are found to increase in number with age

(Belova, 1976; Tamura and Honma, 1977). The presence of macrophages indicates a function associated with cleaning of thymus where lot of necrosis (apoptosis) occurres (Zapata, 1980; Castillo *et al.*, 1990; Pulsford *et al.*, 1991). The macrophages of *Oncorhynchus mykiss* are positive for acid phosphatase, 5'-nucleotidase, and nonspecific esterase (Castillo *et al.*, 1990).

Monocytes (Catillo *et al.*, 1990); multi nucleated giant cells (Pulsford *et al.*, 1991), and melanomacrophages (Gorgollon, 1983; Pulsford *et al.*, 1991) have also been noticed in thymus.

Myoid cells are also noticed in fish thymus like other vertebrates. They appear as large round or oval cells having a light electron dense nucleus and cytoplasm containing myofilaments. In salmons they may assume concentrically arranged muscle fibres and those may be increased with age. The myoid cells are the antigenic source for cell recognition (Henry, 1972; Tamura *et al.*, 1981; Chilmonczyk, 1983; Gorgollon, 1983; Pulsford *et al.*, 1984; Fange and Pulsford, 1985).

Various authors have reported about the occurrence of mast cells, mucus secretory cells inside the thymus of rainbow trout and carp (Kapa and Csaba, 1973; Chilmonczyk, 1983). Thymus in higher vertebrates is protected from ingest of external antigens. Hence antibody-producing cells are not seen in thymus in normal condition. However plasma cells have been reported from the thymus of various fish species (Good *et al.*, 1966, Zapata, 1981; Fange and Pulsford, 1985; Nakanishi *et al.*, 1986; Pulsford *et al.*, 1991). Generally the thymus of fish is impervious to antigens. Though the thymus is present in extreme superficial position in teleosts, the tight junctions of pharyngeal epithelial covering and endothelium of blood vessels effectively protect it from contacting external antigens. Hence, the development of plasma cells is unlikely to take place in normal condition (Chilmonczyk, 1992; Castillo *et al.*, 1998).

There is a little knowledge on the innervation of fish thymus. It is believed that thymus receives innervation from sympathetic system either from fourth or fifth sympathetic ganglion (Lagabrielle, 1983). In mammals sympathetic noradrenergic fibers innervate lymphoid organs, the T and B-lymphocytes functions are mediated by neuroendocrine peptides. Hence it can be assumed that a nerve fibre plays potential link between nervous system and immune system (Payan and Goetzi, 1985). In mammals and birds thymus involute on aging and this involution start at the time of sexual maturity. But in fish complete involution rarely occur. Usually connective tissues accumulate during sexual maturity. The stress, seasons and hormones also influence this involution (Lele, 1933; Hafter, 1952; Ghoneum *et al.*, 1982; Cooper *et al.*, 1983; Pulsford *et al.*, 1984; Nakanishi, 1986; Ellsaesser *et al.*, 1988).

2.2.2 Kidney

Lymphohaemopoietic tissue has been used as a criterion for the selection of organ equivalent to the bone marrow in higher vertebrates. Eventhough morphologically identified lymphocytes appeared in the peripheral blood, the lymphohaemopoietic tissue occurred in various locations including both the nephros and the intestinal lamina propria. In the agnatha (neither hag fish nor lampreys) have no true lymphoid organs (Zapata and Cooper, 1990; Ishiguro *et al.*, 1992; Litman *et al.*, 1992; Du Pasquier, 1993; Kronenberg *et al.*, 1994; Zapata *et al.*, 1995). Chondrichthyes are the most primitive fishes to have true lymphoid organs like orbic, cranium, epigonal organ, meninges etc. they can be considered equivalent to the bone marrow of higher vertebrates (Zapata, 1981a; Fange, 1984; Fange, 1987; Mattisson *et al.*, 1990; Zapata *et al.*, 1996). There was no such report indicating the presence of kidney as lymphoid organ equivalent in cartilaginous fish to the bone marrow of higher vertebrates. In contrast to the above, the primitive bony fishes (osteichthyes) are next in evolutionary process and also bear lymphohaemopoeitc tissues in kidney along with many other organs like cranium, heart, olfactory sac, meninges, spiral valve and gonads etc (Jordan and Speidel, 1931; Good *et al.*, 1966;

Bradshow *et al.*, 1969; Minura and Minura,1977; Millot *et al.*, 1978; McKinney *et al.*, 1981; Zapata, 1983; Fange, 1984; Chiba , 1994; Luft *et al.*, 1994; Clawson *et al.*, 1966). While the teleosts, have only kidney which bear lymphohaemopoietic tissues and equivalent to the bone marrow of higher vertebrates (Ellis and de Sousa, 1974; Roberts, 1975b; Al-Adhami and Kunz, 1976; Zapata, 1979, 1981b; Caspi and Avtalion, 1984; Quesada *et al.*, 1990; Zapata and Cooper, 1990; van Muiswinkel *et al.*, 1991, Imagawa *et al.*, 1991; Press *et al.*, 1994).

Teleost kidney consists of two distinct, although structurally similar, segments: anterior, cephalic or head kidney; and the middle and posterior trunk kidney. Both region exhibit haemopoietic role but it is more prominent in the head kidney in which renal function has disappeared (Ellis and de Sousa, 1974; Roberts, 1975b; Sailendri and Muthukkaruppan, 1975; Zapata, 1979, 1981b; Chantanachookhin *et al.*, 1991; Petri-Hanson and Ainsworth, 2001; Roberts and Ellis, 2003; Moore and Hawke, 2004).

The haemopoietic tissue of kidney of teleosts bears close resemblance to the bone marrow of higher vertebrates but differs in having a highly active reticuloendothelial and antibody-producing cells component. In this respect it has functional similarity to the lymph nodes of mammals. In the primitive teleosts, e.g. salmonids, there is a little tissue specialization, but in higher teleosts, e.g. the cyprinids and pleuronectids, a degree of specialization and organization of the haemopoietic tissue is evident (Press and Evensen 1999). The blast cells are situated within a stroma of reticuloendothelial tissue similar to the bone marrow of mammal. Through out the haemopoetic tissue numerous sinusoids lined with endothelial cells are found. Blood from renal portal vein pass through sinuses for filtration. The endothelial cells lining the sinuses are phagocytic and they trap particulate materials and immune complexes. The corpuscles of stannius and adrenal tissue are embedded within the haemopoetic tissue. Another cellular structure, found throughout teleost haemopoietic tissue but not in higher vertebrates, is the melanomacrophage centers (Roberts, 1975b).

Melanomacrophage centers are spherical aggregates of pigment containing cells. They vary in their degree of organization, depending on species. In the lower teleosts they are clusters of dark cells distributed throughout the haemopoietic tissue. The degree of melanization varies with age but at all ages the pigment present is dark brown or black and has all the biochemical and chemical properties of melanin, although not necessarily laid down on the characteristic melanosomes of integumental melanin. In higher teleosts the amount of dark pigment present in melanomacrophage centers of normal fish is usually very small and majority of their pigment being much lighter in colour (Roberts, 1978, 1989; Roberts and Ellis, 2003). Histochemically this is lipofuscin but Edelstein (1971) has produced evidence to suggest very close chemical affinities between lipofuscins or age pigments and melanin. The morphology of the melanomacrophage centers of higher teleosts is much more closely defined. They are usually nodular, with a delicate agryophilic capsule. In many species they are closely applied to vascular channels and may have a collar of lymphocytes and pyroninophilic cells. After intraperitoneal injection of carbon particles the reticuloendothelial cells lining the sinusoids take out the particle and move through haemopoietic tissue to the melanomacrophage centres. The antigen and immunocomplexes are also transported to melanomacrophage centre (Robert, 1975b; Agius, 1985; Press and Evenson, 1999). The haemopoietic tissue contains different developmental stages of lymphocytes and antibody secretory cells.

Although some lymphocyte cell clusters seem to occupy defined area in the kidney of some teleosts (Zapata, 1979), in general, the lympho-haemopoietic cells are scattered at random through out a stroma of histoenzymatically heterogeneous fibroblastic reticular cells (Ellis, 1977; Grace and Manning, 1980; Quesada *et al.*, 1990; Chantanachookhin *et al.*, 1991; Press *et al.*, 1994) and sinusoidal blood vessels (Roberts, 1975b; Roberts and Ellis, 2003), both with phagocytic capacity (Zapata, 1979). Every haemopoietic cell lineage seems to be differentiated from the cell progenitors (Al-Adhami and Kunz, 1976; Zapata, 1981b) by an important lympho- and plasmacytopoietic capacity (Smith *et al.*, 1967; Zapata,

1979, 1981b). Despite the fact some authors have claimed some resemblance of teleost kidney to lymph nodes (Smith *et al.*, 1967) its capacity for housing and differentiating blood cell precursors supports its phylogenetical relationship to the bone marrow of higher vertebrates (Zapata, 1979), and in general, it is considered to be a postembryonic source of haemopoietic stem cells (Al-Adhami and Kunz, 1976).

Ellis and de Sousa (1974) had shown that small lymphocytes in plaice taken from neural hepatic duct and introduced intravenously entered the kidney tissue via thin walled blood vessels embedded in white pulp surrounding melanomacrophage centers. Antigen stimulation leads to pyrinophilic cells proliferate in the haemopoeitc tissue of carp. It is thought a transformation of lymphocytes to plasma cells follow the same path as in mammals. The germinal centers are not seen but pyroninophilic cell appear in melanomacrophage centers. Electron microscopy has revealed the presence of numerous plasma cells in kidney renal haemopoetic tissue and then the role of haemopoeitc tissue (Press and Evenson, 1999).

2.2.3 Spleen

The spleen is a dark red, triangular organ that lies adjacent to the stomach or flexure of intestine, to which it is attached by a ligament (Bond, 1979). Generally it is single organ but some species of teleosts may be divided into 2 or 3 smaller lobes (Robert and Ellis, 2003; Iwama and Nakanishi, 1996).

In agnatha (hagfish and lampreys) there are no true lymphoid organs. A typical lymphohaemopoietic accumulation in wall of plexiform veins along the entire length of gut, tryphlosole, was described as phylogenetic precursor of the spleen (Good *et al.*, 1966). However, ultrastructural analysis confirmed that these regions represent filtering sites in which phagocytic endothelia of blood vessels and /or circulating macrophages trap both antigenic and nonantigenic materials from the pharyngeal lumen (Page and Rowley, 1982; Ardavin and Zapata, 1988). It is found that the lymphohaemopoietic accumulations in tissues of lamprey changes

throughout its complex life cycle. These changes in the location of lymphohaemopoietic tissues indirectly indicate the relevance of inductive cell microenvironment in the functioning of on lymphohaemopoietic organs of primitive vertebrates (Tanaka *et al.*, 1981; Ardavin *et al.*, 1984; Ardavin and Zapata, 1987).

In elasmobranches spleenic primodium appears as an isolated organ along the ventral surface of gut or pancreas (Fange, 1984). The organ is formed of closely joined mesodermal cells with blood sinuses. The mesodermal cells give rise to reticular network that is colonized by lymphoid cells. Lymphoid tissue is formed around spleenic arteries (Navarrao, 1987; Lloyd-Evans, 1993). Spleen is also present in holocephalans and its histological organization is very similar to elasmobranches (Felten *et al.*, 1985; Payan and Goetzl, 1985).

In elasmobranch the arterial blood supply to spleen is achieved by the lienogastric artery originating from dorsal aorta. In splenic parenchyma, this artery divides into smaller branches without anastomosing. The terminal capillary has thick wall and form ellipsoids. The splenic ellipsoids open into spleenic cords that are framed with a network of reticular cells where several kinds of blood cell found. This network is gathered in to the spleenic vein and finally joins hepatic portal system (Fange and Nilsson, 1985). In some elasmobranches ellipsoids are morphologically distinct. The central capillaries have the discontinuous endothelial cell basement membranes and often appeared perforated or loosely connected. This minute space communicates to surrounding macrophages containing sheath. This sheath forms filtered trap for antigens and immune cells. The macrophages are firmly associated with each other by interdigitation and ellipsoid macrophages contain abundant lipid inclusion (Yoffey, 1929; Zapata, 1980). The masses of lymphoid tissue, which are associated with ellipsoid, form the white pulp. White pulp of elasmobranch is abundant with lymphocytes, plasma cells and macrophages. These cells are involved in immunological exchange (Tomonaga *et al.*, 1984; Fange and Nilsson, 1985; Zapata and Cooper, 1990). The primary role of spleen is immune response in

elasmobranches (Morrow, 1978; Zapata, 1980; Tomonaga *et al.*, 1984, 1985, 1992; Pulsford and Zapata, 1989; Van Muiswinkel *et al.*, 1991)

The structural organization of the spleen of *Calamoichthys*, as described by Yoffey (1929), is remarkably primitive. Splenic arteries and veins run parallel to each other within lymphoid tissue surrounded by red pulp .In *Polypterus senegalus*, an elongated, voluminous spleen appears closely associated with the intestine. It is histologically poorly developed with unclearly delimited red and white pulp. Together with lymphocytes, clusters of both mature and immature plasma cells and striking granulopoietic foci occupy the splenic parenchyma.

The spleen of dipnoans is divided into two non-connected areas within the alimentary canal. Saito (1984) described anatomically the correlation between the development of spleen and the arterial blood vessel in the Australian lungfish, *Neoceratodus forsteri* showing relationship between the vascular dynamics of the foregut and yolk sac and formation of spleen. Thus splenic primodium first appears as a mesenchymal condensation supplied by the third and fourth vitelline arteries. Gradually development of spleenic sinuses with primodium and the formation of gastric and arteric splenic portal systems occur, with organ finally growing along the arterial extremity of the spinal valve. In the South American lungfish *Lepidosiren paradoxa*, it forms a compacted organ with in the wall of stroma and anterior part of intestine, in which red and white region appear clearly (Good et al 1966; Fange, 1982). Jordan and Speidel (1931) describe histologically three regions in the spleen *Protopterus ethiopicus*; the central one consisted of lymphoid cells surrounded by the region of active in erythropoiesis, of cell cords and blood sinuses and thin, peripheral capsular zone.

In chondrosteans and holosteans spleen has red and white pulp. The white pulp is formed of lymphoid aggregates found in splenic parenchyma. The white pulp contains lymphocytes and antibody producing cells (Good *et al.*, 1966). Sturgeons have well developed ellipsoids and these ellipsoids have follicle–like

peripheral masses containing lymphocytes, granulocytes, macrophages (Clawson *et al.*, 1966; Fange, 1984).

Numerous studies have focused on the histology of the teleost spleen (Zapata, 1983; Zapata and Cooper, 1990) although there is a little ultrastructural data (Zapata, 1982; Quesada et al., 1990; Zapata and Cooper, 1990; Press et al., 1994).

The lymphoid tissue is poorly developed in teleost spleen. It surrounds small arteries, appears diffuse in the splenic parenchyma, and is related to the so-called melanomacrophage centers. After antigenic stimulation, increased amount of lymphoid tissue does appear, in the spleen of teleosts. The lymphoid tissue of teleosts consists of lymphoid cells, mainly small, medium and large lymphocytes arranged in a supporting reticular cell meshwork. Some recent data suggest that these stromal reticular cells of the teleost spleen represent a truly heterogeneous cell population. The spleens of teleost fishes contain white and red pulp as in mammals, but are relatively undifferentiated and lack germinal centers (Zapata, 1982; Kennedy-Stoskopf, 1993). Red pulp contains mostly erythrocytes and few lymphocytes. White pulp consists of lymphoid tissue in a reticular network found mainly around blood vessels (Anderson, 1974; Zapata, 1982). Quesada *et al.* (1990) reported ultrastructural differences between the reticular cells in the red pulp and those of white pulp of the sea bass spleen, and Press *et al.* (1994) observed variations in the intensity of enzyme reactivity for alkaline phosphatase and 5'-nucleotidase within the spleen, which could reflect differences between distinct reticular cell populations. On the other hand macrophages appear in the both red and white pulp, and in the latter, macrophages-lymphocytes-plasma cell cluster have been demonstrated ultrastructually (Zapata, 1982).

In the teleost spleen, ellipsoidal blood vessels are lesser developed than in the spleen of elasmobranches, but are organized according to the same pattern with terminal capillaries that show a thin endothelial cell layer surrounded by a sheath of reticular fibers and macrophages (Yoffey, 1929). Remarkably, the splenic

arterioles react for non specific esterase and alkaline phosphatase, but this reactivity disappears as the vessels enter the ellipsoids, reflecting the special nature of ellipsoidal endothelium (Press *et al.*, 1994). These authors also analyzed the histoenzymatical pattern of ellipsoidal macrophages from teleost spleen, remarking on its similarities to the marginal zone macrophages of the mammalian spleen. In fact, the marginal zone limiting the red and white pulp is lacking in the teleost spleen, and according to the scanning electron microscopy studies on vascular corrosion casts, the splenic circulation is open to the arterial capillaries that end in the reticular mesh work of the red pulp (Kita and Itazawa, 1990).

Immunological function of spleenic lymphoid tissue has not been worked out fully. Functions of the splenic lymphoid tissue of teleosts remain, however, controversial. The role of splenic lymphoid tissue in antigen processing seems to be certain. The spleen plays role in antigen processing, antigen binding and /or antibody –production. Antibody secreating cells have been detected in spleen, and trout splenocytes can be stimulated by LPS, PPD, and Con A (Zapata, 1983; Zapata and Cooper, 1990), indirectly suggesting the presence of T-like and B-like cells in the teleost spleen. Splenectomy has no effect on the humoral responses to BSA in some teleosts (Ferren, 1967) although in other species the spleen apparently represents a major lymphoid organ (Yu *et al.*, 1970). Tatner (1985) found a preferential migration of trout thymocytes in to the spleen, a fact previously reported by Ellis and de Sousa (1974) in the plaice, and there was a greater involvement of carp spleen in secondary immune responses. Primary intraperitoneal administration of Human γ-globulin (HCG) in saline or FCA induced few changes in the spleen. In contrast, a secondary immunization with HCG in FCA, but in saline, generated numerous pyroninophilic cells closely associated with splenic ellipsoids. More recently, large clusters of Ig-positive cells have been observed in *Salmo salar* 3 months after vaccination (Press *et al.*, 1994), and increased numbers of pyroninophilic cells appear around ellipsoids and melanomacrophages centers after primary and secondary immunization (van Muiswinkel *et al.*, 1991). The spleen is a primary organ where erythrocytes, neutrophils and granulocytes mature and are

processed or stored (Anderson, 1974). The spleen is also involved in haematopoiesis, the clearance of macromolecules, antigen degradation and processing, and antibody production (Dalmo *et al.*, 1997). Spleenic lymphoid tissue is poorly developed and immune functions of splenectomized fish are not altered (Zapata, 1982). Ferren (1967) reported that splenectomy had no effect on the humoral responses to some teleosts. Yu *et al.* (1970) opinioned the spleen was a major lymphoid organ in fish. Thymocytes of trout and plaice preferentially migrate into spleen (Ellis and de Sousa, 1974; Tatner, 1985). It was also shown the spleen in carp was involved in secondary immuno-responses after intraperitoneal administration of Human γ-globulin (HCG). There was no change in spleen but on secondary immunization numerous pyroninophilic appeared in spleenic ellipsoids. In *Salmo salar* large clusters of Ig-positive cells have been observed after vaccination, pyroninophilic cells appear around ellipsoids and melanomacrophages centers (van Muiswinkel *et al.*, 1991; Press *et al.*, 1994).

2.2.4 Gut-associated lymphoid tissue

All vertebrates, including agnatha, contain lymphoid cells isolated in the lamina propria and the intestinal epithelium, but well organized lymphoid aggregates appear for the first time in chondrichthyes (Fichtelius *et al.*, 1968; Tomonaga *et al.*, 1986; Hart *et al.*, 1988; Zapata and Cooper, 1990; Zapata *et al.*, 1996). Apart from considering species specific variation in size, the histological organization of fish gut-associated lymphoid aggregates is similar in all species studied. It consists of un-encapsulated lymphoid accumulations that contain mainly lymphocytes, macrophages and plasma cells as well as different types of granulocytes (Zapata et al., 1996; Moore and Hawke, 2004). Teleosts have a common mucosal immune system, with lymphoid aggreagates associated with the gut, reproductive tract, skin, and gills (Zapata *et al.*, 1996; Moore and Hawke, 2004).

2.3 INFLAMMATION

Inflammation is a process that begins following injury (Robert, 1978; Suzuki and Iida, 1992) to tissues and ends with healing. This process common to all vertebrates and is principally a protective response to the affected tissue. Although occasionally such response may initiate severe disease in certain site (Roberts, 1978). It is characterized by a series of changes that take place following injury.

In fishes mainly three major typical changes happen during inflammation (Suzuki and Iida, 1992; Secombes, 1996) following injuries due to various means of natural or experimental stimuli (Roberts, 1989; Suzuki and Iida, 1992; Woo, 1992; Secombes, 1996). Those three important changes are: (a) vasodilatation and vascular permeability (Weinreb, 1959; Finn and Nielsen, 1971a, b; Suzuki and Hibiya, 1981, 1983), (b) leucocyte migration and removal of debris (Mawdesley-Thomas Bucke, 1973; Iger and Abraham, 1990), and (c) resolution and healing (Phromsuthirak, 1977; Sommer and Bartos, 1981; MacArthur *et al.*, 1984).

The acute inflammation response occurs whenever pharmacodynamic amines (histamine or 5-hydroxytryptamine) are released, from mast cells or complement Mast cells are present in almost all tissues (Roberts, 1978). Principally injuries reported are of two types: natural and induced stimuli. Varieties of materials are used for the response and those include injection of phlogistic agents such as bacteria (Finn and Nielsen, 1971a, b; Phromsuthirak, 1977; MacArthur *et al.*, 1984; Sommer and Bartos, 1981; Park and Wakabayashi, 1989), exposure to metazoan parasites (Pulsford and Mattews, 1984; Hoole, 1994), subcutaneous inoculation with fungi, intrapulmonary stimulation with carbon and latex (Suzuki and Hibiya, 1986), and wounding (Roberts, 1989; Suzuki and Iida, 1992; Woo, 1992) and others (Finn and Nielsen, 1971a, b; MacArthur *et al.*, 1984; Sovenyi and Baros, 1986). The amines released following injuries cause capillary dilatation, increase blood flow to affected area and even increase lumen diameter of capillary fenestrae to allow the largest serum protein molecules such as fibrinogen and immunoglobulins to exude

into the tissues. Leucocytes actively migrate through the fenestrae to enter the affected tissue. In all cases a common acute inflammatory response is elicited, characterized by neutrophilia and monocytosis in the blood, and an accumulation of neutrophils and macrophages at the site of injury or infection (Roberts, 1989; Suzuki and Iida, 1992). The cellular response like a typical biphasic leucocyte migration, controlled by chemical mediators, with neutrophils arriving at the site of inflammation more quickly than macrophages are noticed (Ellis, 1986; Roberts, 1989; Suzuki and Iida, 1992). Once the leucocytes and proteins reached in affected area they perform their duties. Neutrophils and macrophages remove debris by phagocytosis while serum protein molecules neutralize the toxic effect of the injurious agents. Few authors claimed presence of basophils and eosinophils in the inflammatory response. This has not been positively ascertained in any fish (Suzuki and Iida, 1992; Moore and Hawke, 2004). They are mostly absent from inflammatory area though some authors attributed antiparasitic role for them (Cone and Wiles, 1985; Reimschuessel *et al.*, 1987; Powell *et al.*, 1993). The eosinophilic granular cells (EGCs) present in many tissues have got a significant role in inflammation. They are analogous to mast cells in mammals. Degranulation of eosinophilic granular cells occurs after an injection of bacterial exotoxin this is followed by transcends appearance of histamine in blood and widespread vasodilatation. Leucocyte migration, infiltration, and phagocytosis are followed by tissue repair (Roberts, 1978; Suzuki & Iida, 1992; Secombes, 1996; Dalmo *et al.*, 1997; Moore and Hawke, 2004). The acute inflammatory response in fish results in hemorrhagic liquefaction rather than contained suppuration (Ellis, 1981; Moore and Hawke, 2004). This response is more damaging to host tissue, and fish may be able to respond in this way due to the greater regenerative power to replace damaged tissue by new cells (rather than scar tissue replacement in mammals) (Ellis, 1981).

If the inflammatory stimuli persist following the acute inflammatory response, a chronic inflammatory response may follow. Granuloma formation is a typical chronic inflammatory response in fish. Granulomas are organized collections of macrophages and fibrous tissue stroma. As the granuloma progresses, the

macrophages aggregate and transform into epitheloid cells or multinucleate giant cells (Smith, 1964; Roberts and Bullock, 1976; Timur *et al.*, 1977; Secombes, 1985; Roberts, 1989; Suzuki and Iida, 1992; Woo, 1992; Secombes, 1996), and extensive melanization and fibrosis occurs. Granulomas may displace or isolate the surrounding host tissue resulting in tissue atrophy (Ellis, 1981; Secombes, 1996; Moore and Hawke, 2004).

2.4 PHAGOCYTOSIS

Phagocytosis is a defense function in higher vertebrates. Phagocytes recognize and eliminate non self-material that enter and proliferate in the body. The vascular system enables phagocytes to reach distant tissue/sites of injury.

Though several types of cells show ability to ingest particulate materials in fish, the mononuclear cells / monocytes and granulocytes, are considered as major phagocytes (MacArthur and Fletcher, 1985; Rowley *et al.*, 1988, Secombes and Fletcher, 1992; Secombes, 1996). There is confusion about identification of granulocytic phagocytes. In carp there are two granulocytes series of which one is phagocytic (MacArthur and Fletcher, 1985; Sovenyi and Kusuda, 1987). Some authors are of the view that neutrophils are poor phagocytes (Griffin, 1983; Ellis, 1976; Ellis *et al.*, 1976; Suzuki, 1984; MacArthur and Fletcher, 1985; Ellis, 1986; Roberts, 1989; Wood *et al.*, 1986). With the development of the light and electron microscopy, the phagocytosis and intracellular killing ability of fish neutrophils became more clear (Ainsworth, 1992). Carp neutrophils require some maturation to become active phagocytes. In channel catfish, though neutrophils are able to phagocytosis but they are unable to kill internalized *Edwardsiella ictaluri* (Waterstrat *et al.*, 1991). In *Oreochromis mossambicus* neutrophils were weakly phagocytic but some individuals showed high phagocytic activity (Manoj, 1996).

Macrophages in fish are abundant in various tissues. They are mostly found in peritoneal spleen, gills and atrium of the fish heart. Small number of phagocytes is found in blood (Ellis *et al.*, 1976; Chilmonczyk and Monge, 1980; Roberts 1989; Nakamura *et al.*, 1991; Manoj, 1996). Monocytes are precursor of the tissue macrophages (Roberts, 1989).

Hyder *et al.* (1983) emphasized that the peripheral blood eosinophil of elasmobranches were not phagocytic. Few studies highlighted phagocytic nature of teleost eosinophils (Ezeasor and Stokoe, 1980; Huizinga, 1980; Bodammer, 1986; Doggett *et al.*, 1987; Doggett and Harris 1989; Manoj, 1996). The functional role of basophil is not clear in fishes (Ainsworth, 1992; Manoj, 1996).

2.4.1 Mechanisms of phagocytosis

Phagocytosis process consists of sequence of events namely signal, pursuit, surface recognisation, adherence and engulfment. This is followed by internalization and healing (Babior, 1984; Blazer, 1991).

Internal killing involves the production of super oxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl (OH) and hypohalite ions (XO). The energy for generation of these anions is obtained by exonous monophosphate activity. Such energy production process is called respiratory brust and this has been reported in fish (Kanner and Kinsella, 1983; Chung and Secombes, 1988; Nagelkerke *et al.*, 1990; Sharp and Secombes, 1993; Iiba and Wakabayashi, 1995; Manoj, 1996).

Oxygen independent microbicidal mechanisms consisting of cationic proteins, lysozymes, lactoferrin and proteolytic enzymes were also reported (Rowley *et al.*, 1988; Secombes and Fletcher, 1992).

Both *in vivo* and *in vitro* methods are used to study pahgocytosis in fish. Phagocytes are isolated from peripheral blood, peritoneal cavity, and spleen and anterior kidney. The single discontinuous density gradient method can be used to isolate leucocytes from blood (Blaxhall 1985; Waterstrat *et al.*, 1988). It is possible to separate leucocytes from the anterior kidney, peritoneal cavity and spleen of fish using multiple layer discontinuous density gradients. With this method, granulocytes and mononuclear cell can be separated (Braun-Nesje *et al.*, 1981; Sakai 1984; Chung and secombes, 1988; Anderson, 1992).

Direct and indirect method can be used to evaluate *in vitro* phagocytosis and subsequent intracellular killing. The most commonly used evaluation technique is phagocytic index (Blazer, 1991; Manoj, 1996). The other direct methods are flow cytometry, colony forming unit (CFU), fluorescent techniques and bacterial thin layer method (Olivier *et al.*, 1986; Thuvander *et al.*, 1987; Nakayasu *et al.*, 1995). In fish the commonly used particles to study *in vitro* phagocytosis are yeast, sheep red blood cells, and bacteria, and latex beads (Sovenyi and Kusuda, 1987; Thuvander *et al.*, 1987; Saggers and Gould, 1989; Sheldon and Blazer, 1991). In the indirect methods, estimation of enzymes activity and products of respiratory burst or both are used. These include nitro blue tetrazolium (NBT) test, conversation of phenol red by hydrogen peroxide (H_2O_2), and chemiluminescence assay etc. (Scott and Klesius, 1981; Chung and Secombes, 1988; Secombes *et al.*, 1988; Seeley *et al.*, 1990; Anderson *et al.*, 1991).

2.5 BLOOD SERUM PROTEINS

Many authors studied haematological parameters like electrolytes their appropriate ratio, cholesterol and urea nitrogen level, total protein, albumin, globulin and albumin-globulin ratio and different enzymes (Misra *et al.*, 2006; Misra, *et al.*, 2006a, b). They showed interspecific differences between species, between hybrids and their parents. Very recently, Sifa *et al.* (2000) also worked out the biochemical components of serum and total protein, albumin, and globulin and A/G ratio in

different strains of males and females of *Oreochromis niloticus.* They exhibited that there is significant difference among strains.

Plasma protein concentrations are lower than in man (7 g/l), values from 1.68 to 6.19 g/l have been recorded in different species of teleosts (Roberts and Ellis, 2003). Ellis (2003) further pointed out that the immunological and other functions of the proteins are broadly similar to those in higher vertebrates but many interspecific differences remain to be investigated.

2.5.1 Serum protein profile

Electrophoresis makes it possible to obtain a quantitative analysis of the serum constitutes in an electric field. Electrophoresis separation can be achieved in various media (e.g. filter paper, agar gel and starch gel, etc) (Schaperclaus, 1986). The paper electrophoresis enables the identification of five protein fractions (albumin, α1, α2, β and μ -globulins), 2-3 lipoprotein fractions (α1, α2, β) in blood serum of fish (Kulow, 1966, Schaperclaus, 1986). Cellulose acetate electrophoresis and polyacrylamide electrophoresis enable a further separation of protein fractions of serum into 8-16 components (Balsano and Rasch, 1975; Kirsipuu, 1975; Ingram and Alexander, 1977; Schaperclaus, 1986). Paper electrophoresis according to Dittmer (1956) is suitable for routine electrophoresis investigations of serum proteins in fish (Schaperclaus, 1986).

All physiological and pathophysiological changes in fishes are first manifested in liver, kidney and spleen, therefore in the blood and last in the body musculature (Schaperclaus, 1986). An estimate of the total proteins and in different fractions is of particular importance; since under physiological conditions, the protein substances are subjected to a rigid neurocentral regulation, where by the rate of decomposition exactly correspond to the rate of formation. Therefore any decrease in serum protein level certainly indicates protein deficiency. This protein deficiency can be seen by comparing the total protein level and albumin and globulin fractions of

normal healthy fish vs affected one (Schaperclaus, 1986). Normal electrophoretic protein fractions of important cultured teleosts were worked out (Kulow, 1966). In other finding, there is a remarkable variation in total protein level, albumin and globulin fractions and their percentage between normal and infected fishes (Kulow, 1967). Their finding was supported by many workers later (Schaperclaus, 1986).

Variations in blood serum proteins are noticed in many of interspecies and intraspecies in fishes. These variations are due to their genetic constitution and the influence of physiological and environmental factors. Investigation related to sexual difference of serum protein patterns are also been studied by many others. They have used polyacrylamide, agar, starch gel electrophoresis and immunological methods for estimation of different protein fractions. Harris (1974) and Perrier *et al.* (1974) reported the presence of a dense-staining new component in the serum of mature lamprey and some teleosts. Vanstone and Ho (1961) identified slow fraction called serum vitellin in the serum of maturing female coho salmon (*O. kisutch*) which was absent in the plasma of males, immature, spawning and spent females. Many investigations have attempted to study the changes occurring in the proteins patterns of teleost ovary during its development and to identify, purify and characterize the vitellogenin-derived yolk proteins in variety of fishes (Mano and Lipmann, 1966; Hara *et al.*, 1980; Wallace and Selman, 1981; Riazi *et al.*, 1988; Gopalkrishnan, 1991).

2.6 CELL MEDIATED IMMUNE (CMI) RESPONSES

Specific immune responses independent of antibodies are collectively called cell-mediated immunity. The cells responsible for such immunity were mediated by lymphocytes (Mitchison, 1953) later proven such cells are mainly T (thymus–derived) lymphocytes (Tizard, 1992). Apart from cell-mediated immune responses T lymphocytes (T cells) are also required for production of antibody responses (Arkoosh and Kaattari, 1991; Tizard, 1992).

T cells originate in thymus. Two types of T cells are identified based on their functions. Helper T (T_h) cells enhance immune responses. Cytotoxic T (T_c) cells destroy foreign cells and suppress responses of other B and T cells (Chilmonczyk, 1992; Kennedy-Stoskopf, 1993).

In general, two principal ways are used for estimation of cell-mediated immunity. *In vivo* measurements in fish include histocompatibility responses and hypersensitivity responses. Histocompatibility is determined by assessing the immune response to grafts (Mori, 1931; Rijkers and Van Muiswinkel, 1977; Botham *et al.*, 1980; Tatner, 1990; Hansen *et al.*, 1993; van Muiswinkel, 1995; Manning and Nakanishi, 1996). They have found that antibodies are not involved in graft rejection. Lymphocytes and macrophages are cells that invade the graft.

2.6.1 Delayed (type IV) hypersensitivity response

Delayed (type IV) hypersensitivity (DTH) responses are noticed when certain antigens injected intradermally to animals. The inflammation occurs in skin is delayed for 24 to 48 h. This delay is mainly duration of interaction between the antigen and T cells (Tizard, 1992). Fish show typical delayed hypersensitivity reactions against *Mycobacterium* and parasitic antigens (Manning and Nakanishi, 1996). Rainbow trout (*Oncorhynchus mykiss*) immunized with complete Freund's adjuvant containing *Mycobacterium tuberculosis* or *Mycobacterium salmoniphilum* produced typical DTH skin reactions (Bartos and Sommer, 1981). Activated macrophages were noticed at site of inflammation using silicon skin windows. Besides teleosts, the DTH reactions to mycobacterial antigens are also been demonstrated in lamprey (*Petromyzon marinus*), elasmobrachs (guitar fish, *Rhinobatos productus*, and horned shark, *Heterodontus franciscii*), and chondrosteans (paddle fish, *Polyodon spathula*) (Finstad and Good, 1964; Tam *et al.*, 1976).

Attempts other than *Mycobacterium* as antigens for DTH skin reaction were shown encouraging results. Delayed type hypersensitivity reaction in trout against *Yersinia ruckeri* (Gram-negative bacterium that causes enteric redmouth disease) exhibited possible cross reactivity (Stevenson and Raymond, 1990). Similarly the channel catfish (Ictalurus *punctatus*) immunized with bacterium, *Flexibacter columnaris*, or with channel catfish virus, *Herpesvirus ictaluri*, was failed to show DTH responses while challenged (Pauley and Heartwell, 1983).

Delayed type hypersensitivity reactions against parasitic antigens have been demonstrated in both primitive bony fish and teleosts. Hypersensitivity reactions against metazoans like *Ascaris* in the bowfin,*Amia calva* was studied by Papermaster *et al.*,1964 and against protozoan hemoflagellate *Cryptobia* in rainbow trout by Stevenson and Raymond,1990 and Thomas and Woo ,1990.

Histological findings of DTH reaction in fungal infected rainbow trout against the spores of *Ichthyophonus* (McVicar and McLay, 1985) and against injected viable *Saprolegnia* hyphae (Bly and Clem, 1992) exhibited granulomata formation.

2.6.2 Proliferative responses to mitogens

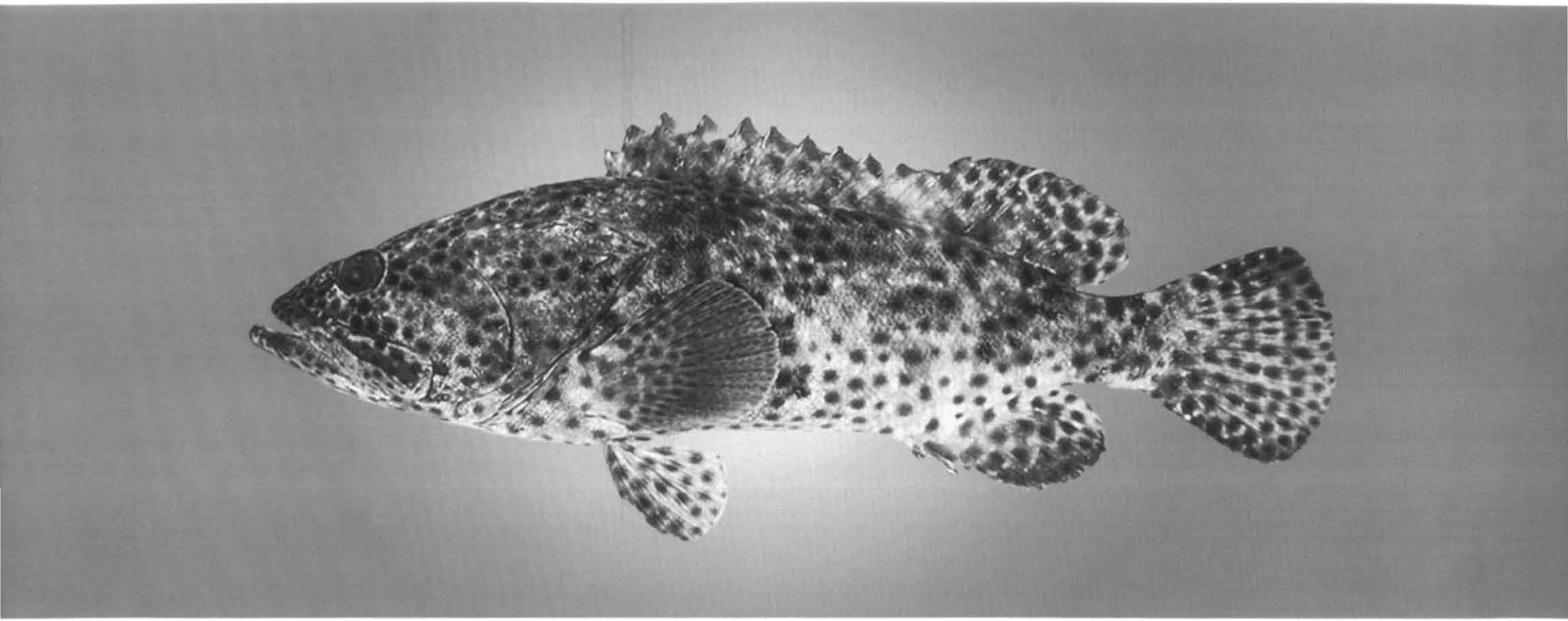
Proliferative responses to B- and T- cell mitogens can be measured *in vivo*. In mammals, the plant lectins phytohemagglutinin (PHA) and concanavalin (Con A) are specific for carbohydrate moieties on T cells. Appropriate doses of these T-cell mitogens induce the proliferation of T cells but not the B cells. Mitogens for B cells, such as lipopolysaccharide, result in the proliferation of B cells but not T cells (Manning and Nakanishi, 1996).

Like mammals, fish also can respond to T- and B-cell mitogens. Mitogen induced proliferation in fish have been studied extensively in teleosts compared to primitive bony fishes (DeKoning and Kaattari, 1992). Cooper (1971) conducted experiments on primitive lampreys (agnathans). In his study, lymphocytes separated from ammocoete larvae responded to PHA *in vitro* by production of blast cells. The blast cell production suggested existence of T-like cells. Similar studies in cartilaginous fish (nurse shark, *Ginglymostoma cirratum*) using Con A and PHA again yielded a T-cell like response (Lopez *et al.*, 1974; Sigel *et al.*, 1978; Pettey and McKinney, 1981; Haynes and McKinney, 1991).

In teleosts, the ability to respond to T –cell and B-cell mitogens was first studied (Etlinger *et al.*, 1976), and then after many followed (Rowley *et al.*, 1988). These include reports on the channel catfish (Sizemore *et al.*, 1984), carp (Caspi and Avtalion, 1984), rainbow trout (Warr and Simon, 1983; Tillit *et al.*, 1988; Reitan and Thuvander, 1991), and Atlantic salmon, *Salmo salar* (Smith and Braun-Nesje, 1982; Reitan and Thuvander, 1991). Documentation of *in vitro* culture requirements for the each species has been important (Rosenberg-Wiser and Avtalion, 1982; Faulmann *et al.*, 1983; DeKoning and Kaattari, 1991). For example, the addition of fetal bovine serum to the culture medium as the serum supplement did not show promising results. The T-cells performed better in salmonoids if homologous plasma (DeKoning and Kaattari, 1992) and in channel catfish if monocytes (Sizemore *et al.*, 1984) are used. Sizemore *et al.* (1984) have demonstrated that the sIg^{+ve} cells responded to LPS irrespective to presence or absence of monocytes, where as sIg^{-ve} population remained unresponsive to either LPS or Con A unless the accessory cells were present. A similar response of sIg^{-ve} cells to LPS was reported by DeLuca *et al.* (1983) using pronephric leucocytes of rainbow trout. In rainbow trout, thymocytes proliferate in response to PHA but not to LPS (Reitan and Thuvander, 1991). Essentially similar results were obtained for channel catfish using the thymus from fish about 14 months old and stimulating with Con A (Ellsaesser *et al.*, 1988). Accessory cells (monocytes) were required for the T-cell activation and either autologous or allogenic cells could be used. Again, there was a small response in

some cases to LPS. However, this dose not detracts from the general conclusion thymocyte are Ig^{-ve} cells that display a T –cell like response to mitogens. Contrary to above, thymus can play a minor role as a residence for small population of B cells (Manning, 1994).

MATERIAL AND METHODS



Greasy Grouper *Epinephelus tauvina* (Forsskal, 1775) (Photo courtesy : www.fishbase.org)

3. MATERIAL AND METHODS

Experiments were conducted at the Marine hatchery complex of Central Marine Fisheries Research Institute (CMFRI), Cochin. Studies on histomorphology, ultrastructure and blood and serum samples were carried out in the Pathology laboratory at CMFRI, Cochin.

3.1 EXPERIMENTAL FISH

Greasy grouper, *Epinephelus tauvina* collected from Chinese dip nets from the bar mouth area of Cochin backwaters, were used in the present study. They were transported to CMFRI marine hatchery complex in plastic cans containing clean, aerated seawater of salinity 15 ± 5 ppt. Healthy fishes without injuries were selected for the studies. The total length and body weight of the experimental fishes varied from 142 - 320 mm and 30 - 420 gm respectively. The fishes were given a formalin bath at a concentration of 100 ppm for 30 min before releasing them into tanks for maintenance.

3. 2 MAINTENANCE OF FISH

One ton capacity oval FRP tanks having built in biological filters were used for the maintenance of fish. Fishes were released into the tanks holding seawater of salinity 15 ± 5 ppt following formalin bath. Tanks were provided with 24 h continuous aeration. Fishes were fed *ad libitum* with small frozen whole prawns once a day. Uneaten feed was removed after 2 h followed by siphoning of faecal matter and other sediments. Fifty percent of the water was replaced every week.

3.3 BLOOD COLLECTION FROM FISH

Blood samples collected from fishes were pooled and used for the characterization of leucocytes. Fishes were anaesthetized using clove oil (100 ppm) prior to bleeding. The anticoagulant used was Heparin sodium (BEPARINE, Biological E. Limited, Hyderabad, India). The blood samples were collected by heart puncture using sterile heparinised syringe with 20-gauge needle and

transferred to heparinised vials. The blood thus collected was kept immediately in icebox for further analysis.

3.4 SERUM COLLECTION FROM FISH

Blood intended for serum separation was allowed to clot by keeping the vials in a slanting position at room temperature for 1 h after which it was kept at 4 ^{0} C for the clot to contract. The clot was then removed by centrifugation at 3000 rpm for 15 min. The sera thus collected from fishes were stored at – 20 ^{0} C for further studies.

3.5 ISOLATION OF BLOOD LEUCOCYTES

Leucocytes from whole blood were separated by density gradient centrifugation as per Blaxhall (1985). Four ml of leucocyte separation medium (Histopaque-1077, Sigma) was taken in 10 ml sterile glass centrifuge tube. Two ml of extracted blood was carefully layered over the separation medium and centrifuged for 30 min at 400g in a refrigerated centrifuge. A gray ring at the interface of blood plasma and the separation media was observed. The supernatant (blood plasma) above the gray ring was carefully removed using a sterile Pasteur pipette and then discarded. The leucocyte layer (gray ring) was aspirated without disturbing the separation medium with another sterile Pasteur pipette.

3.6 HAEMATOLOGY

Fish were grouped in to three based on size and body weight for haematological estimations. Erythrocyte and leucocyte counts were estimated as per Schaperclaus (1986).

3.6.1 Erythrocyte count

Diluted blood was loaded to the Neubauer counting chamber and erythrocytes were counted in the five group squares (1group square = 16 small squares). The total Erythrocytes Count (EC) was determined as follows:

$$EC/mm^3 = \frac{\text{Erythrocytes in eighty small squares}}{\text{Area} \times \text{depth of chamber} \times \text{dilution}}$$

3.6.2 Leucocyte count

The leucocytes were counted in the four large squares at the four angular points of the Neubauer counting chamber. The total Leucocyte count (LC) was calculated as follows:

$$LC/mm^3 = \frac{\text{No. of leucocytes in four squares of 1 } mm^2}{\text{Area} \times \text{depth of chamber} \times \text{dilution}}$$

3.7 TOTAL PROTEIN AND ALBUMIN

Total protein (Gornall *et al.*, 1949) and albumin were determined by the Biuret method and BCG dye binding method respectively using a commercially available diagnostic kit (Sigma Diagnostics, India, Pvt. Ltd. Product no. 72111, 72121). The sera collected from fish and kept in – 20 ^{0} C, as described previously under isolation of serum, were used for this studies. The absorbance was measured using Genesys-10 UV/ VIS Spectrophotometer (Thermospectronics, USA) at 555 nm for total proteins and 630 nm for albumin. Globulins were determined by subtracting the albumin value from total protein value.

3.8 DIFFERENTIAL LEUCOCYTE COUNT

Differential leucocyte count was estimated as per Schaperclaus (1986).

3.8.1 Preparation of blood smear

Blood samples collected by heart puncture using sterile heparinised syringe with 20-gauge needle were immediately used for preparation of blood smears. Fresh drop of blood was placed on a clean, degreased glass slide. Cover slip was held in a slanted position and glided on the slide towards the blood drop until the drop has spread along the edge of cover slip. The cover slip was then moved immediately in an inclined position (at an angle of 45^0) in the direction away from the blood drop across the slide for the spread. The smears were then air dried.

3.8.2 Fixation and staining

Air dried blood smears were fixed in methanol for 3 min at room temperature. The fixed smears were stained in 20-30 drops (0.5-0.8 ml) of filtered May-Grunwald solution for 3 min and then equal quantity of distilled water was added into it for 1 min. Whole solution was decanted after 4 min and then counterstained in dilute Giemsa solution (10 drops in 10 ml of distilled water) for 15-20 min. The slides were washed vigorously in distilled water and allowed for drying. The slides were mounted in DPX, dried and then observed under light microscope.

3.8.3 Leucocyte count and photography

Stained blood smears were observed under an oil immersion objective to count different leucocyte populations. A total of 200 leucocytes were counted. The percentages of each leucocyte population against the total leucocytes counted in different fishes were estimated. Photomicrographs of leucocytes were also taken.

3.9 CYTOCHEMICAL STUDIES ON LEUCOCYTES

3.9.1 Acid phosphatase

Blood smears were prepared on clean, dry glass slides and then allowed for air drying. Air-dried blood smears were fixed in formalin-acetone (20% formalin in 50% acetone) for 1 min at 0 °C, followed by rinsing in running tap water

(Sanders, 1974). The fixed smears were stained in freshly prepared and filtered acid phosphatase substrate solution containing Fast Blue BBN (Annexure I) at 37 °C for 1 h, followed by rinsing and air drying. The stained smears were then counterstained in 0.1% neutral red solution for 3 min followed by drying. The smears were then cleared in xylene, mounted in DPX and observed under microscope.

3.9.2 Peroxidase

Air dried smears prepared as above were fixed in 10 % alcoholic formalin for 1 min and later rinsed in distilled water for 15-20 sec. The wet slides were stained in myeloperoxidase incubation mixture, pH 6 ± 0.5 (Annexure II) for 30 sec followed by washing in running tap water and drying. The slides were counterstained in Giemsa for 10 min. The smears were then cleared in xylene, mounted in DPX and observed under microscope.

3.9.3 Periodic Acid Schiff (PAS)

Air-dried smears were fixed in methyl alcohol. Fixed slides were placed in 0.5% periodic acid solution for 7 min and rinsed in distilled water. The slides were kept in reducing bath (Annexure III) for 1 min and rinsed in distilled water. The slides were then placed in Schiff's reagent (Annexure III) for 30 min followed by washing in running tap water for 5 to 10 min and then rinsing in distilled water. The slides were stained in filtered celestine blue solution (Annexure III) for 10 to 15 min and rinsed with distilled water, and air-dried. The slides were then cleared in xylene, mounted in DPX and observed under microscope.

3.9.4 Sudan black B

Air-dried smears were fixed in formalin vapour for 10 min and rinsed in running tap water. The slides were stained in working Sudan black B solution (Annexure IV) for 1 h. The slides were washed with 70% alcohol for 2 to 3 min followed by washing in running tap water for 2 to 3 min and drying. The slides were counterstained in May-Grunwald-Giemsa, cleared in xylene, mounted in DPX and observed under microscope.

3.10 TRANSMISSION ELECTRON MICROSCOPY OF LEUCOCYTES

Ultrastructure studies were carried out in a Hitachi H-600 Transmission Electron Microscope (HITACHI Ltd, Tokyo, Japan). The tissue preparation and processing was done as per Dawes (1988). Leucocyte pellet was prepared and characterized based on the ultrastructural features.

3.10.1 Preparation of blood leucocyte pellet

The separated leucocyte layer was transferred to a clean, sterilized 10 ml centrifuge tube that contained three times the volume of chilled (4°C) Hanks balanced salt solution (HBSS) (Hi Media) with 2% fetal calf serum (Hi Media: 100 I.U./ml penicillin, 100 µg /ml streptomycin and 100 I.U./ml heparin). The homogeneous suspension was then centrifuged at 100g for 5 min. The supernatant was drawn in a Pasteur pipette and then discarded. The pellet was resuspended in chilled HBSS. The above step was repeated thrice until the leucocyte pellet was free of Histopaque.

3.10.2 Fixation

Leucocyte pellet was immediately transferred to 3% chilled glutaraldehyde solution in sodium cacodylate buffer, pH 7.2. The pellet was fixed for 6 h at 4 °C. The fixed pellet was washed in 0.1M sodium cacodylate buffer three times (30 min each), and then kept overnight in buffer. The pellet was post fixed in 1% osmium tetroxide (in cacodylate buffer) for 1 h at 4 °C. The leucocytes were again washed 3 times (30 min each) in buffer and centrifuged as above after each wash to get a pellet. Agarose gel (2.5 % concentration) prepared in cacodylate buffer, pH 7.2, at 50°C was added to the pellet. The contents were mixed thoroughly and allowed to solidify. The solidified agarose cone was cut into small 1 mm^3 pieces.

3.10.3 Dehydration and embedding

Dehydration of the agarose cubes containing the leucocytes were carried out in ascending grades of acetone (Annexure V) at 4 °C and embedded in Spurr's resin as per the method described by Spurr (1969).

3.10.4 Sectioning and staining

Ultra thin sections (60-90 nm) were taken in LKB Nova Ultra microtome (LKB - Producter AB, Sweden) using glass knives. These sections were lifted on to the matted surface of copper grids (mesh size - 300) and allowed to dry. The dried sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshall, 1965), dried and observed under transmission electron microscope at 50 KV accelerating voltage.

3.11 HISTOLOGY OF LYMPHOID ORGANS

Tissues of thymus, kidney and spleen were taken for histological investigations.

3.11.1 Fixation

The tissues were fixed in 10 % neutral buffered formalin overnight. The tissues were scored with a sharp blade for easy penetration of the fixative.

3.11. 2 Tissue processing and microtomy

Tissues were processed (Bullock, 1978) before paraffin embedding in an automatic tissue processor (Leica, Germany) and sections of 5 µm thickness were cut in a semiautomatic rotary microtome (Leica, Germany). The tissue processing schedule followed is given in Annexure VI.

3.11. 3 Staining

Paraffin sections were cleared in xylene, hydrated with descending grades of alcohol and stained in haematoxylin after which they were passed through acid alcohol, Scott's tap water and eosin (Culling *et al.*, 1985). The stained sections were dehydrated in ascending grades of alcohol, cleared in

xylene and mounted with DPX. Mounted sections were observed under light microscope (Leica, Germany) and photographed under different magnifications.

3.12 SPECIAL STAINING OF LYMPHOID ORGANS

Tissues of thymus, kidney and spleen were taken for special staining. Special stains like Reticulum-Gomori, Trichrome-Masson and Verhoeff's Van Gieson, were done as per Melby and Altman (1974). Fixation, tissue processing and microtomy were carried out as described above.

3.12.1 Reticulum – Gomori

Tissues were deparaffinised in xylene and brought to water. The tissues were oxidized in 0.5% potassium permanganate solution for 1 min and washed in running tap water for 2 min. The tissues were then decolourised in 2% potassium metabisulphite for 1 min followed by washing in tap water for 2 min. The tissues were placed in 2% ferric ammonium sulphate for 1 min followed by washing in running tap water for 2 min. The tissues were rinsed twice in distilled water for 30 sec each and then in ammoniacal silver solution for 1 min. The tissue sections were rinsed in distilled water for 20 sec. The sections were placed in reducing solution for 3 min followed by washing in running tap water for another 3 min.

Sections were toned in 1% gold chloride solution for 10 min until the brown tones have disappeared. The tissue section were rinsed in distilled water and placed in 2% potassium metabisulphite solution for 1 min. The sections were transferred immediately to 2% sodium thiosulphate for 1minute and then allowed for washing in tap water for 2 min. The sections were counterstained lightly with Mayer's haematoxylin for 20 to 30 sec. The counterstained sections thus placed in running water appeared blue. The slides were dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX.

3.12. 2 Trichrome - Masson

Sections were deparaffinised in xylene, brought to water and stained in preheated Harris haematoxylin for 5 min at 58°C. The tissue sections were rinsed in 95% alcohol and then differentiated in picric alcohol for 10 min followed by washing in running tap water for 10 min.

The tissues were counterstained in Ponceau red for 5 min followed by rinsing in distilled water and differentiated in 1% phosphomolybdic acid for 5 min. The tissue sections were placed in Aniline blue Masson for 2 min and rinsed in tap water and again placed in 1% acetic acid for 5 min. The slides were dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX.

3.12. 3 Verhoeff's Van Gieson

Sections were deparaffinised in xylene and then brought to water followed by staining in Verhoeff's haematoxylin solution for 15 min to 1 h and then washing in distilled water. The tissue sections were differentiated in 2% ferric chloride and washed in water. The slides were placed in 95% alcohol for 5 min, washed in distilled water for 5 min and then counterstained in Van Gieson solution for 1 min. The slides were dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX.

3.12. 4 Photomicrography

Mounted sections were observed under light microscope (Leica, Germany) and photographed under different magnifications.

3.13 TRANSMISSION ELECTRON MICROSCOPY OF LYMPHOID ORGANS

Ultrastructure studies were carried out in Hitachi H-600 Transmission Electron Microscope (HITACHI Ltd, Tokyo, Japan). The tissue preparation and processing was done as per Dawes (1988). Lymphoid organs like thymus, kidney and spleen were studied for ultrastructural details.

3.13.1 Fixation

One mm cubes of tissues from lymphoid organs were excised from anaesthetized fishes and immediately transferred to chilled buffered 3 % glutaraldehyde solution in sodium cacodylate buffer, pH 7. 2. The tissues were fixed for 6 h at 4 °C. The fixed tissues were washed in 0.1M sodium cacodylate buffer three times (30 min each) and kept overnight in the buffer. The tissue cubes were post fixed in 1% osmium tetroxide (in cacodylate buffer) for 1 h at 4 °C and followed three washings in cacodylate buffer.

3.13.2 Dehydration and embedding

Dehydration of tissues were carried out in ascending grades of acetone at 4 °C and embedded in Spurr's resin as per the method described by Spurr (1969).

3.13.3 Sectioning and staining

Ultra thin sections (60-90 nm) were taken in LKB Nova Ultra microtome (LKB - Producter AB, Sweden) using glass knives. These sections were lifted on to the matted surface of copper grids (mesh size - 300). The sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshall, 1965), dried and observed under transmission electron microscope at 50 KV accelerating voltage.

3.14 INFLAMMATORY RESPONSE

Healthy fishes, weighing 100 -150 gm maintained in tanks were selected for studies on the inflammatory responses.

3.14.1 Administration of Freund's complete adjuvant

The healthy fishes were anaesthetized in clove oil at a concentration of 100 ppm. They were injected with 0.5 ml Freund's complete adjuvant (FCA) (Sigma-aldrich, USA), in the intramuscular region on the left side, below the dorsal fin. The fishes thus injected with FCA were released back to tanks for rearing.

3.14.2 Histology

Tissues were excised from the site of injection after 24 h. Fixation, processing, microtomy and staining were carried out as described under histology. Mounted sections were observed under light microscope (Leica, Germany) and photographed under different magnifications.

3.15 *IN VIVO* PHAGOCYTOSIS

Healthy fishes, (100-150 gm), maintained in laboratory were selected for studies on phagocytic activities. Kidney, spleen, peritoneum and heart were used for this investigation.

3.15.1 Administration of Indian ink

Indian ink (Camlin Ltd., India) was diluted in normal saline (0.85% NaCl) in the ratio 1:10. The suspension was thoroughly mixed and centrifuged for 10 min in 3000 rpm. Supernatant was separated and autoclaved for 15 min at 15 pounds (120 °C) and allowed for cooling in room temperature. Diluted Indian ink thus prepared was used as the source of colloidal carbon particles for this study.

Fishes were anaesthetized in clove oil at a concentration of 100 ppm. They were injected with 0.5 ml diluted Indian ink intraperitoneally. Injected fishes were released back to tanks for rearing.

3.15.2 Histology

Tissues were dissected out after 24 hr post injection and fixed in 10% neutral buffered formalin overnight. The tissues were scored with a sharp blade for easy penetration of the fixative. Tissue processing, microtomy and staining were carried out as described under routine histology.

3.15.3 Photography

Mounted tissues were observed under light microscope (Leica, Germany) and photographed under different magnifications.

3.16 *IN VITRO* PHAGOCYTOSIS

In vitro phagocytosis studies were conducted as per Glick *et al.*, 1964.

3.16.1 Blood leucocyte suspension

Blood leucocytes isolated by density gradient centrifugation as per Blaxhall (1985), were washed thoroughly in HBSS to remove histopaque. The leucocytes free from histopaque were resuspended in chilled HBSS at concentration of 2×10^6 cells/ ml and kept in refrigerator.

3.16.2 Yeast cell suspension

Commercial yeast, *Saccharomyces cerviceae* was used for the preparation of yeast cell suspension. Yeast grains were powdered, transferred into sterile test tube and autoclaved for 20 min at 120 $^{\circ}$ C (15 lbs). The autoclaved yeast powder was suspended aseptically in cold HBSS. The yeast cell suspension thus prepared was adjusted to 4×10^7 cells/ ml and stored at room temperature.

3.16.3 Phagocytosis of yeast cells

One hundred microlitres (0.1 ml) of leucocyte suspension was placed on a clean grease-free sterile glass coverslip and incubated inside a humid chamber containing 5% CO_2 for 1 h at room temperature. The coverslip was washed gently in cold HBSS to remove all non-adherent cells. Two hundred microlitre of yeast cell suspension was added to the coverslip coated with adhering leucocytes (to get phagocyte and yeast ratio, of 1:40) and then incubated inside a humid chamber containing 5% CO_2 for 1 h. The coverslip coated with leucocytes and killed yeast cells was washed twice with cold HBSS and air dried. The air dried coverslip was fixed with methanol for 1 min and stained with dilute Giemsa (1:10) as per the method of Pappenheim (Schaperclaus, 1986). The stained coverslip was cleared in acetone, and mounted with DPX.

3.16. 4 Phagocytic index

The cells coated on coverslip were observed under oil immersion objective. Total 200 cells (with or without ingested particles) were counted and phagocytic index (PI) was calculated using formula (Blazer, 1991) given below.

$$\text{Phagocytic Index (PI)} = \frac{\text{No. of ingested yeast cells x 100}}{\text{Total no. of cells counted}}$$

3.17 SKIN SENSITIVITY TO PHYTOHAEMAGGLUTININ (PHA)

Skin sensitivity to the T-cell mitogen, PHA was determined according to Rajan *et al.* (1986). One hundred microlitre of PHA-M was injected intradermally at the caudal peduncle of the fishes and skin thickness at the caudal peduncle was measured using a screw gauge after 24, 48, 72 and 96 h.

3.18 ROSETTE FORMATION TECHNIQUES

Leucocytes were separated by density gradient technique as described above in section 3.4. They were washed in HBSS three times. The cells were suspended in HBSS containing 10% foetal calf serum. The cell suspention was made at concentration of 2×10^6 cells/ ml. A 2% suspension of rabbit erythrocytes was made in normal saline. Equal quantity of the leucocyte suspension and the RBC suspension was mixed and incubated at 10^0 C for 12h. The drops of the suspension were examined under high power microscope and estimated the number of rosette forming cells (Steward, 2002).

3.19 NATIVE – POLYACRYLAMIDE GEL ELECTROPHORESIS OF SERUM

Native Polyacrylamide gel electrophoresis (PAGE) was carried out as per Davis (1964). Sera collected from fishes, (30-420 gm), were used in this investigation.

3. 19. 1 Separating gel and stacking gel

Separating gel (7.5%) and stacking gel (3.5%) necessary for native-PAGE were prepared as per composition given in Annexure VII.

3. 19. 2 Casting of gel

Gel cassette was set with clamps after proper cleaning and three sides were sealed with Vaseline. The separating gel was poured into the cassette below the well. To correct the meniscus few drops of butanol was poured above the separating gel. Gel was allowed to polymerize for 15-30 min. Then butanol was poured off and washed with double distilled water. Stacking gel was poured above the polymerized separating gel. A comb was placed above the stacking gel without entrapping any air bubble and allowed to polymerize for 15-30 min. The polymerized gel was kept for 10 min inside the refrigerator. The comb was removed from polymerized gel. The polymerized gel with the cassette was fixed inside the electrophoretic unit to connect the upper and lower reservoir of the buffer.

3. 19.3 Loading of sample

Serum was diluted with double distilled water in 1: 5 ratio. Samples and loading buffer (Annexure VII) were mixed in the ratio of 1:1. Accordingly 25 µl of sample was mixed with 25 µl of loading buffer and loaded into the wells. The sample was electrophoresed for 5-6 h in refrigerated conditions at a constant current of 3 mA per well. Then the gel was kept in the fixative made of methanol and acetic acid (aqueous solution containing 15% methanol and 7% acetic acid) for 1 h.

3.19.4 Staining, destaining and documentation

Fixed gel was stained with comassie brilliant blue R-250 stain till the development of bands (1-1.5 h). Then the gel was kept in destaining solution (aqueous solution containing 10 % methanol and 7% acetic acid) overnight. After complete destaining, the gel was photographed and used for further analysis.

3. 20 STATISTICAL ANALYSIS

Data from the studies on haematological parameters and serum factors were subjected to statistical analysis. Mean value of each parameter was compared using correlation and ANOVA and R* C X 2 test (SPSS version 14).

RESULTS

4. RESULTS

4.1 HAEMATOLOGY

Total erythrocyte and leucocyte counts were determined from the fishes of length 142- 320 mm and body weight 30 – 420 gm. The three groups of fish were viz. less than 100 gm, 100 -200 gm, and above 200 gm. The values of total erythrocyte and leucocyte count of all three groups are presented in table 1.

4.1.1 Total erythrocyte count

The total erythrocyte count of the fishes varied from 0.80 × 10^6 /mm^3 to 13.5 × 10^6 /mm^3 with mean count 4.42 × 10^6/mm^3 ± 0.69.The erythrocyte counts in relation to the body weight were shown in figure 1. The total erythrocyte counts between three groups were not significantly different. The erythrocyte counts did not show correlation with the body weight.

4.1.2 Total leucocyte count

The total leucocyte count varied from 32.5 × 10^3/mm^3 to 150 × 10^3/mm^3 and the mean count was 72.75 × 10^3 /mm^3 ± 0.86. The leucocyte counts in relation to the body weight were shown in figure 2. The total leucocyte count increased with body weight and showed high degree of correlation ($p < 0.05$) and the counts did not show significant difference between the three groups.

4. 2 SERUM FACTORS

Serum factors studied include total protein, albumin, globulin and albumin: globulin ratio. The values of serum proteins from all the three groups viz. less than 100 gm, 100-200 gm, and above 200 gm are presented in table 2.

Table 1. Total erythrocyte and leucocyte counts in *E. tauvina*.

Total erythrocyte count				**Total leucocyte count** [2]		
Groups	Body Weight (in gm)	Counts ($\times 10^6$/ mm^3)		Groups	Body Weight (in g)	Counts ($\times 10^3$/ mm^3)
Gr-I	30	0.92		Gr-I	30	38.0
	45	0.80			45	34.5
	50	5.02			50	33.0
	50	3.74			50	52.5
	55	2.89			55	59.5
	70	3.07			70	34.5
	85	2.62			85	106.5
Gr-II	100	7.28		Gr-II	100	70.5
	110	6.99			110	46.0
	115	5.41			115	81.5
	133	5.11			133	130.0
	145	2.92			145	93.0
	162	5.18			162	77.0
Gr-III	215	3.40		Gr-III	215	32.5
	230	5.06			230	84.0
	290	1.04			290	62.0
	410	4.68			410	150.5
	420	13.50			420	124.0

Groups	Erythrocyte Counts (Mean ± standard error)	Leucocyte counts (Mean ± standard error)
Gr-I	2.72 $\times 10^6$/ mm^3 ± 0.57	51.21$\times 10^3$/ mm^3 ± 0.99
Gr-II	5.48 $\times 10^6$/ mm^3 ± 0.64	83.00 $\times 10^3$/ mm^3 ± 0.11
Gr-III	5.53 $\times 10^6$/ mm^3 ± 0.21	90.60 $\times 10^3$/ mm^3 ± 0.86
Whole	4.42 $\times 10^6$/ mm^3 ± 0.69	72.75 $\times 10^3$/ mm^3 ± 0.86

1. The mean values from different groups of erythrocyte and leucocyte counts were compared by one-way ANOVA.
2. The leucocyte counts showed high degree of correlation (* $p < 0.05$) with body weight.

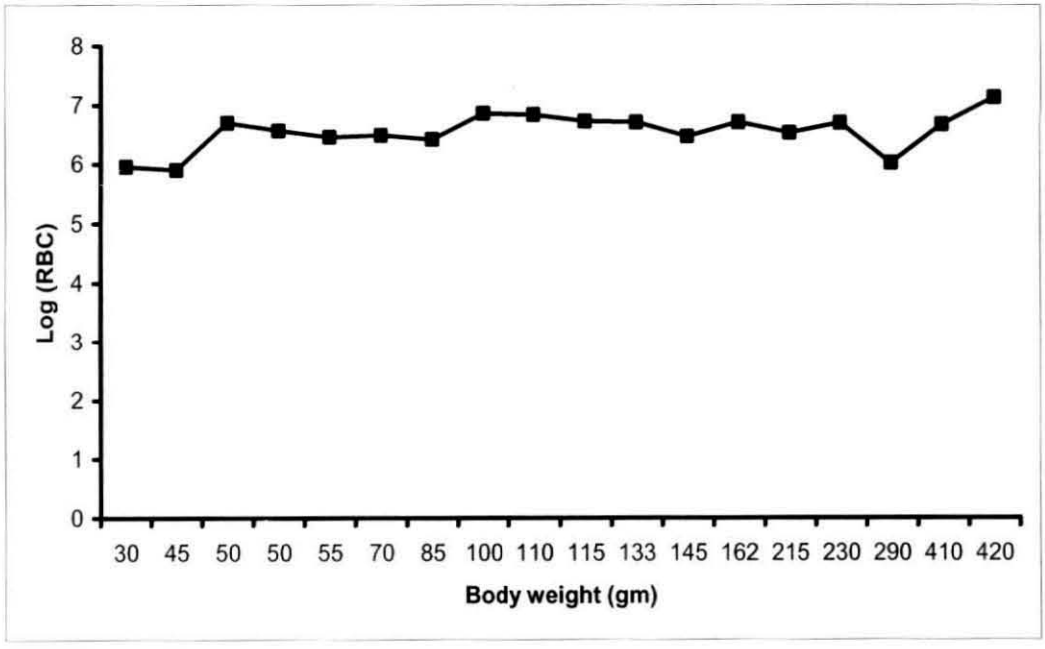


Figure 1. Total erythrocyte counts of *E. tauvina* in relation to the body weight.

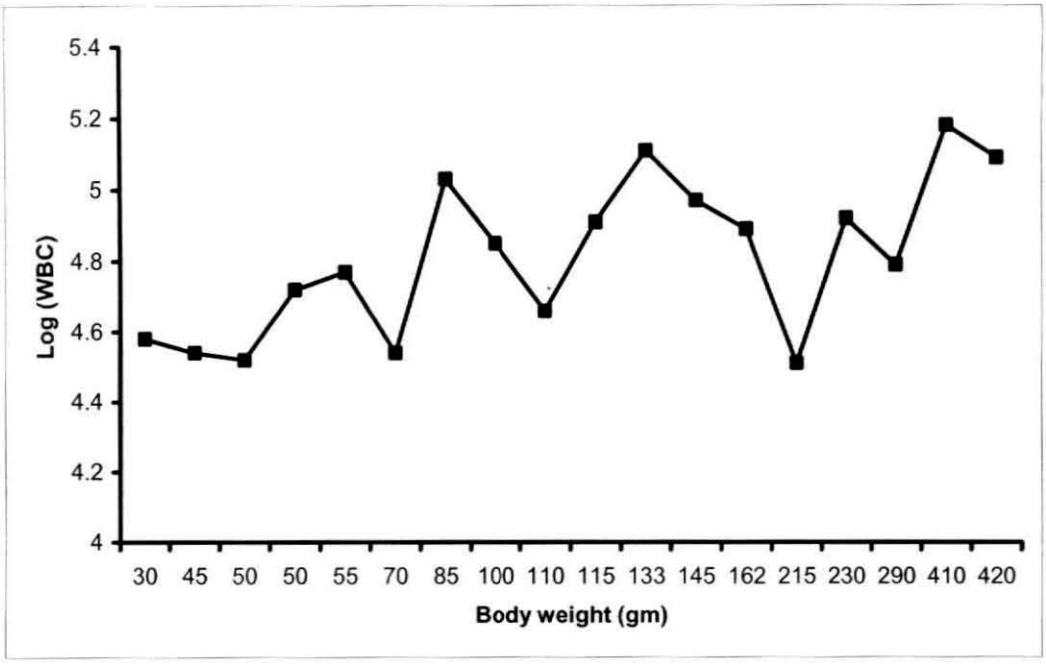


Figure 2. Total leucocyte counts of *E. tauvina* in relation to the body weight.

Table 2. Serum protein values of *E. tauvina*.

Group	2 (*) Body Weight (gm)	Total protein (gm %)	Albumin (gm %)	Globulin (gm %)	2 (*) A/G ratio
Gr-I	30	0.786	0.008	0.778	0.010
	50	1.810	0.025	1.768	0.024
	50	1.810	0.042	1.768	0.024
	55	0.859	0.05	0.809	0.061
	55	3.329	0.067	3.262	0.020
	55	0.859	0.130	0.729	0.178
Gr-II	100	3.018	0.050	2.968	0.016
	100	1.975	0.050	1.925	0.025
	110	2.780	0.025	2.755	0.010
	110	2.652	0.135	2.517	0.053
	133	3.329	0.840	2.489	0.337
	145	3.310	0.085	3.225	0.026
	145	1.353	0.093	1.26	0.073
Gr-III	215	3.164	0.432	2.732	0.158
	215	0.165	0.008	0.157	0.050
	215	2.835	0.008	2.827	0.002
	230	3.109	0.118	2.991	0.040
	290	1.463	0.365	1.098	0.332
	345	0.402	0.080	0.312	0.288
	410	0.841	0.120	0.721	0.166

Mean serum protein values

Groups	Total protein (mean ±s.error)	Albumin (mean ± s. error)	Globulin (mean ±s. error)	A/G ratio (mean ± s. error)
Gr-I	1.57 ± 0.40	0.05 ± 0.01	1.51 ± 0.40	0.08 ± 0.02
Gr-II	2.63 ± 0.27	0.18 ± 0.11	2.44 ± 0.25	0.09 ± 0.02
Gr-III	1.71 ± 0.49	0.16 ± 0.06	1.55 ± 0.47	0.12 ± 0.02
Whole	1.99 ± 0.24	0.14 ± 0.04	1.85 ± 0.23	0.09 ± 0.01

1. The mean values from different groups of serum proteins were compared by one-way ANOVA.
2. The A/G ratio showed high degree of correlation (* $p < 0.05$) with body weight.

4.2.1 Total protein

The total serum protein values showed wide variation and ranged from 0.16 gm % to 3.32 gm % with mean 1.99 ± 0.24 %. The total protein values did not depict any relationship with the body weight of the fish. The protein values, in different groups of fish did not show significant variation. The total serum protein values in relation to the body weight were shown in figure 3.

4.2.2 Total albumin

The total serum albumin values showed wide variation ranged from 0.008 gm% to 0.840 gm% with mean 0.14 ± 0.04 %. The serum albumin did not show any relationship with body weight. The serum albumin values in relation to the body weight were shown in figure 4.

4.2.3 Total globulin

The total serum globulin values showed wide variation ranged from 0.157 gm % to 3.270 gm % with mean 1.85 ± 0.23 %. The globulin values did not show any relationship with body weight. The serum globulin values in relation to the body weight were shown in figure 5.

4.2.4 Albumin / globulin ratio (A/G ratio)

The A/G ratio showed variation from 0.01 to 0.34 with mean 0.09 ± 0.01. The A/G ratio showed high degree of correlation ($p < 0.05$) with the body weight of fish. The A/G ratios in relation to the body weight are shown in figure 6.

4.3 Serum protein profile

The serum proteins were separated into 8- 10 fractions. Of these 4-5 fractions were prominent and readable in densitometer (plate 1). The fractions were representative of various serum proteins and those were 1^{st} fraction (21.23 ± 1.15 %), 2^{nd} fraction (19.03 ± 1.59 %), 3^{rd} fraction (20.45 ± 0.71%), 4^{th} fraction (23.17 ± 2.01%) etc. (table 3 and figure 7). The probabily the fast moving fractions contain albumin

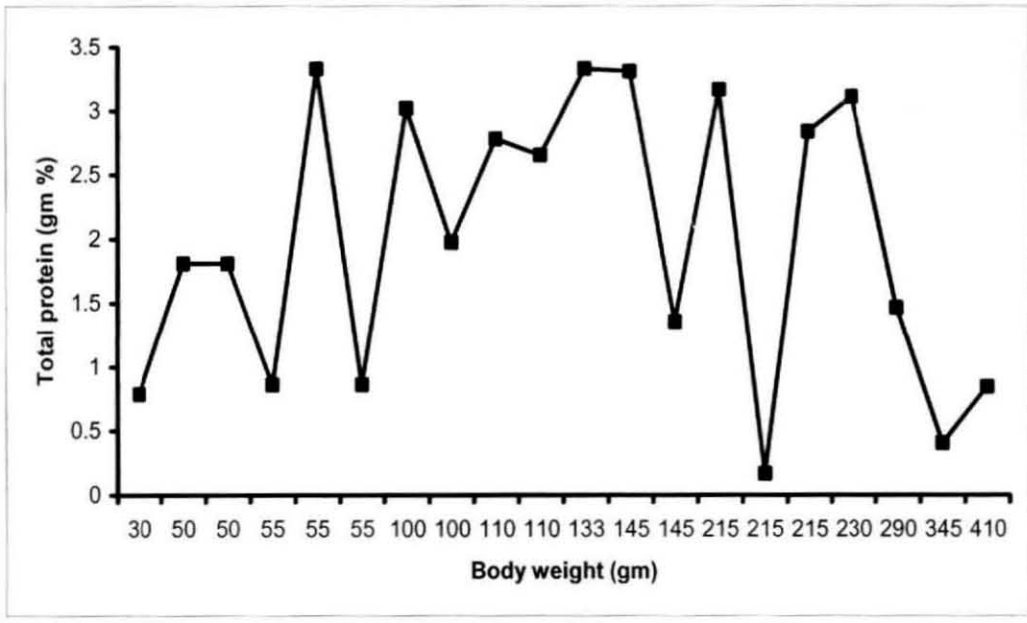


Figure 3. Total serum protein values of *E. tauvina* in relation to the body weight.

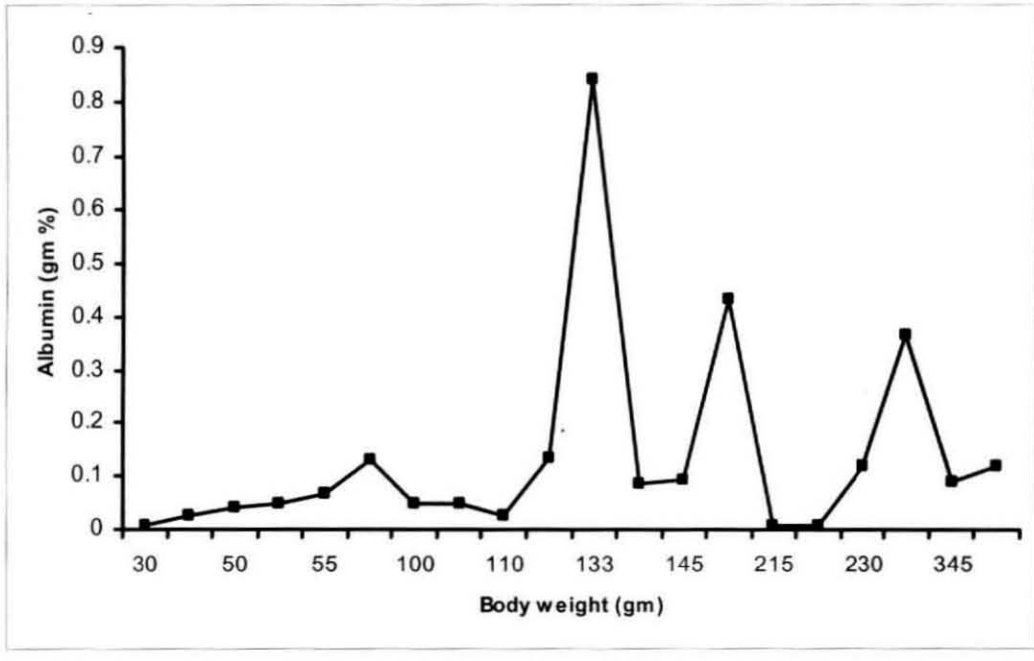


Figure 4. Serum albumin values of *E. tauvina* in relation to the body weight.

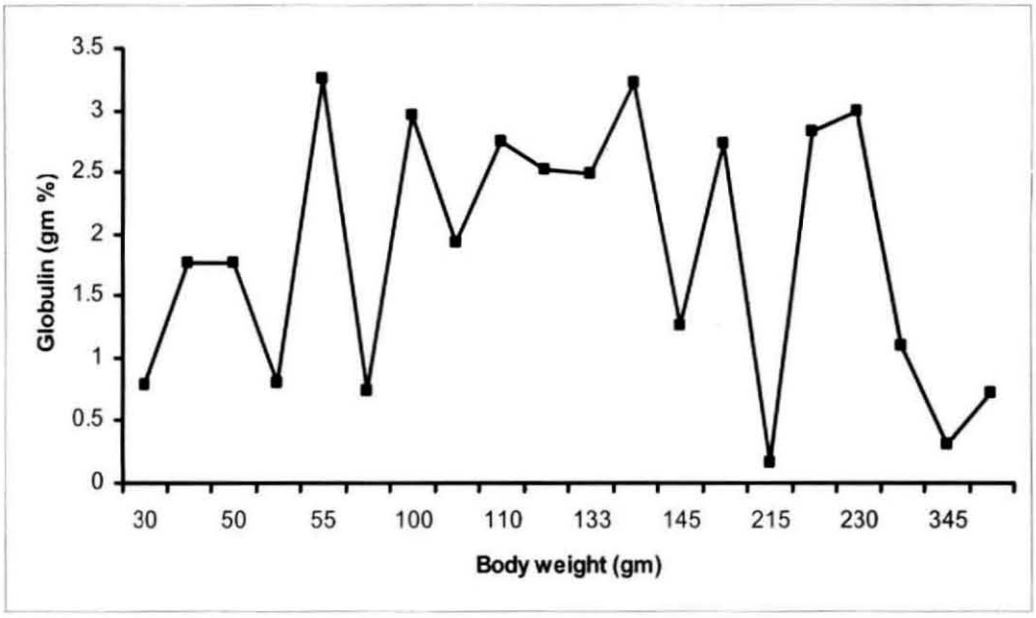


Figure 5. Serum globulin values of *E. tauvina* in relation to the body weight.

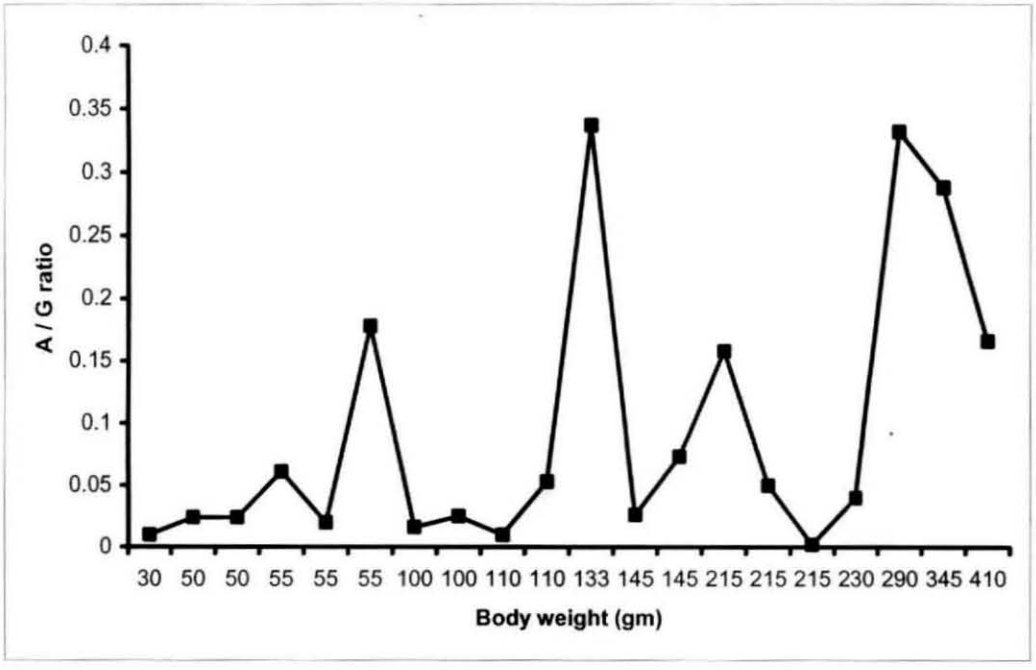


Figure 6. Serum A / G ratio of *E. tauvina* in relation to the body weight.

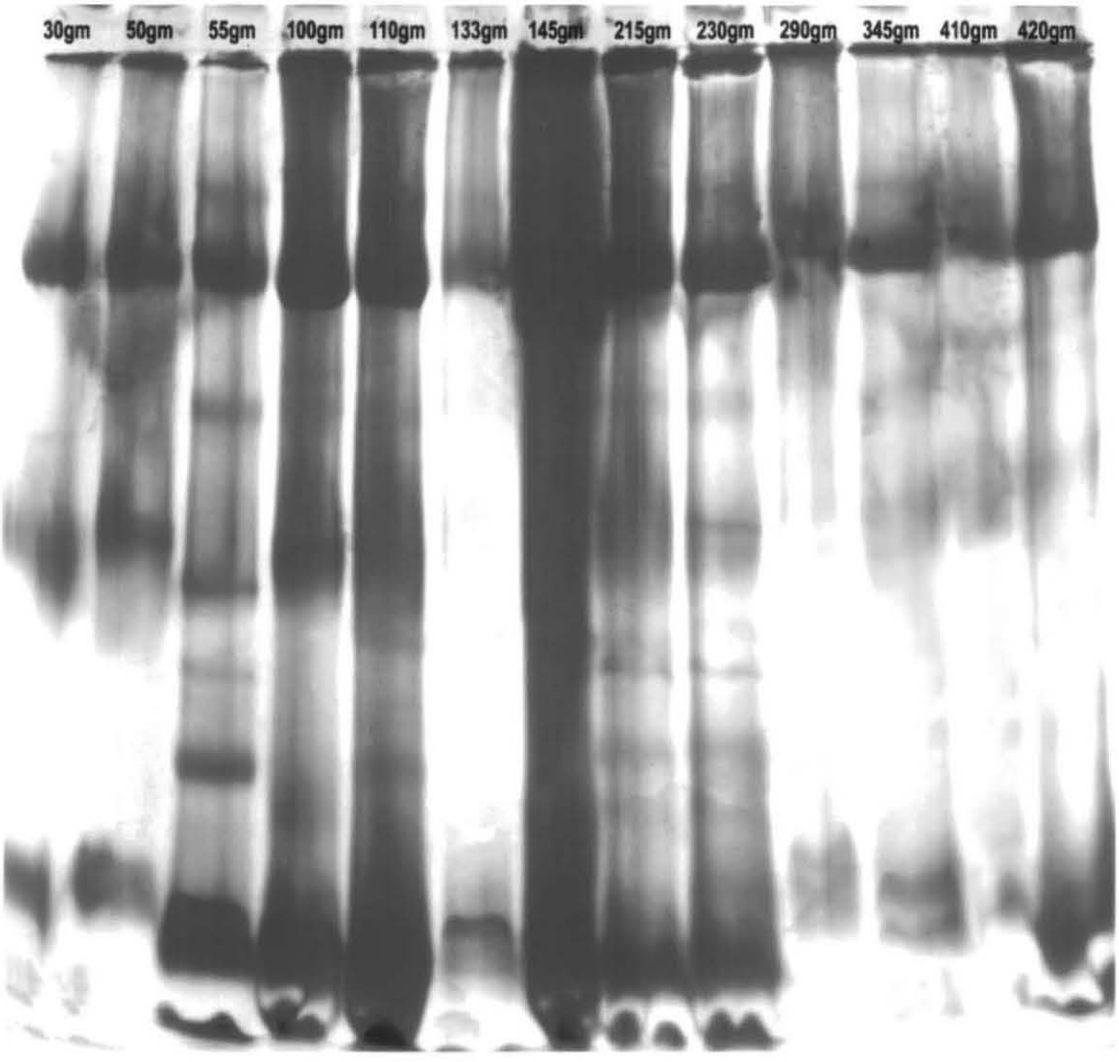


Plate 1: Electrophoretic serum protein profiles of E. *tauvina* in relation to the body weight.

Table 3. Electrophoretic serum protein profile in *E. tauvina*.

SERUM PROTEIN PROFILE					
Body weight (in gm)	1 st fraction (in %)	2 nd fraction (in %)	3 rd fraction (in %)	4 th fraction (in %)	5 th fraction (in %)
30	19.37	21.12	20.32	38.48	-
50	15	16	19	16.5	33
55	19	22	20.2	20.2	19
100	21.3	25	22	30.45	-
110	21.56	26.62	22	28	-
133	31.14	15.86	18.66	31.8	-
145	22.31	25.36	23.5	28	-
215	21.45	26.46	18.21	32.22	23.4
230	15	19.1	15.4	26.2	21
290	23.1	12.6	25	17.35	21.7
345	22.6	11.8	21.7	14.5	22.9
410	19.4	14.04	21.69	14.4	21.42
420	24.8	11.45	18.1	13.0	24.4

Types of fractions	Mean ± standard error
1 st fraction	21.23 ± 1.15
2 nd fraction	19.03 ± 1.59
3 rd fraction	20.45 ± 0.71
4 th fraction	23.17 ± 2.01
5 th fraction	22.10 ± 0.60

The mean percentage of serum protein fractions and the body weight showed high degree of significant ($p < .01$) in one-way ANOVA test.

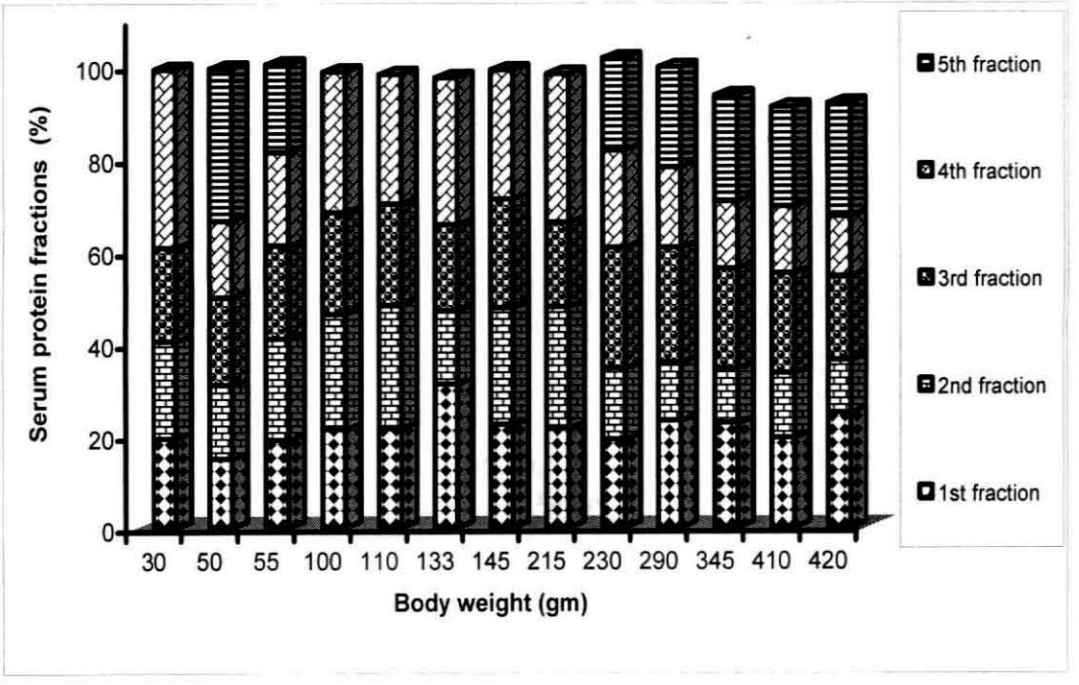


Figure 7. Electrophoretic serum protein fractions of *E. tauvina* in relation to the body weight.

followed by various fractions of globulins. The last moving fractions may be containing immunoglobulins. The mean percentage of serum protein fractions and the body weight showed high degree of significant difference ($p < .01$).

4.4 LEUCOCYTES

Blood smears prepared from healthy *Epinephelus tauvina* were stained with May-Grunwald and Giemsa. The smears revealed oval shaped erythrocytes. The nucleus of erythrocyte stained pink while cytoplasm appeared pale eosinophilic. The differential counts of leucocytes revealed mainly four types of cells: the granulocytes, large cells monocytes, spindle shaped thrombocytes and basophilic lymphocytes. The percentage of different leucocytes varied significantly ($p < 0.01$) in relation to the body weight of the fish (table 4).

4.4.1 Granulocytes

The differential count revealed the percentage of granulocytes varied between 22 and 37 and the mean percentage was 31.83± 6.48. Majority of cells were unlobed nuclei or indented nuclei while some were lobed nuclei (plates 2a – 2f). The cytoplasm contained granules some of which stained mildly basophilic while others took mild eosinophilic stain. The mildly stained basophilic cells were more in number and they formed 26.66 ± 2.06 % where as 5.16 ± 0 .65 % were having eosinophilic granules in their cytoplasm. (Figure 8).

The cytochemical studies showed many of these cells were positive for PAS and peroxidase and stained with Sudan black. Some were mildly acid phosphatase positive. (Plates 3a - 3i).

4.4.1.1 Ultrastructure

Cells with two types of granules were noticed under transmission electron microscope. In one type, numerous uniformly homogenous electron dense granules were abundant in cytoplasm. Occasionally one or two mitochondria were seen in cytoplasm of the cells while the Golgi apparatus and endoplasmic reticulum were poorly

Table 4. Differential leucocyte counts of *E. tauvina*.

Differential leucocyte counts of *E. tauvina*				
Body Weight (in gm)	Lymphocyte (in %)	Monocyte (in %)	Eosinophils (in %)	Neutrophils (in %)
50	63	5	7	25
85	61	3	4	32
100	55	8	5	32
110	54	11	7	28
215	70	8	3	19
420	68	3	5	24

Mean leucocyte percentage of different classes	
Leucocyte	Mean ± standard error
Lymphocyte	61.83± 2.67
Monocyte	6.33± 1.30
Eosinophil	5.16± 0 .65
Neutrophil	26.66± 2.06

The body weight against various leucocyte counts were compared by R × C, X^2 Contingency test. The counts were significant at 1% level ($p < .01$).

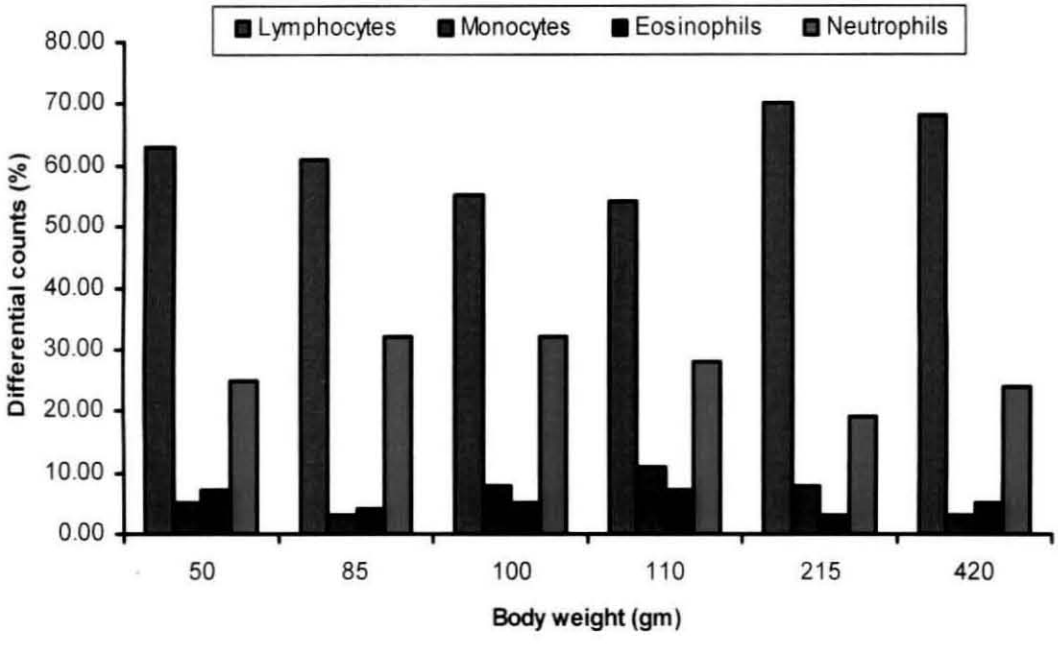


Figure 8. The differential leucocyte counts of *E. tauvina* in relation to the body weight.

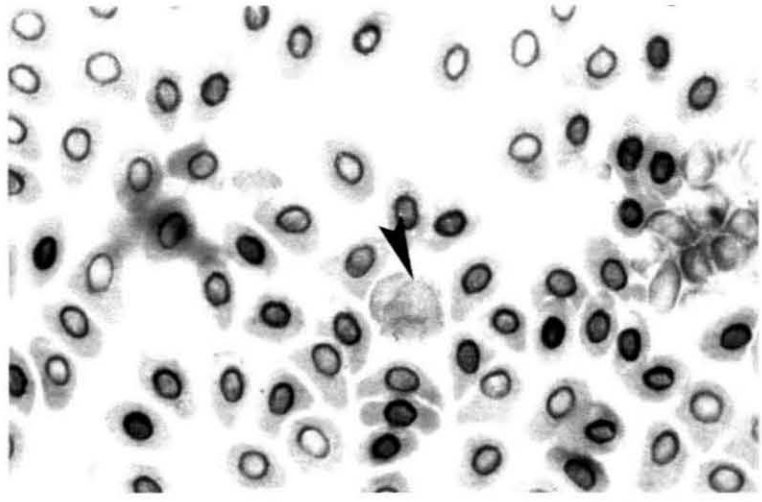


Plate 2a. Granulocyte of the *E. tauvina*. Note the eccentric lobed nucleus (arrowhead) with the blue granules at periphery of the cytoplasm. May-Grunwald and Giemsa stain, 1000 x.

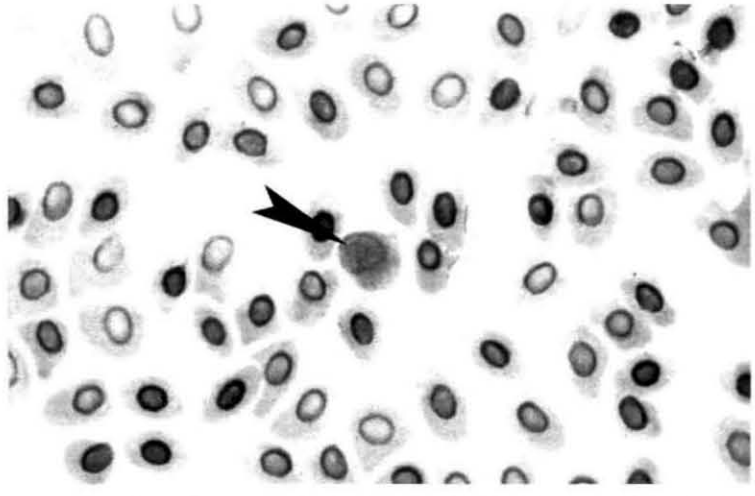


Plate 2b. Granulocyte of the *E. tauvina*. Note the eccentric unlobed nucleus (arrowhead). May-Grunwald and Giemsa stain, 1000 x.

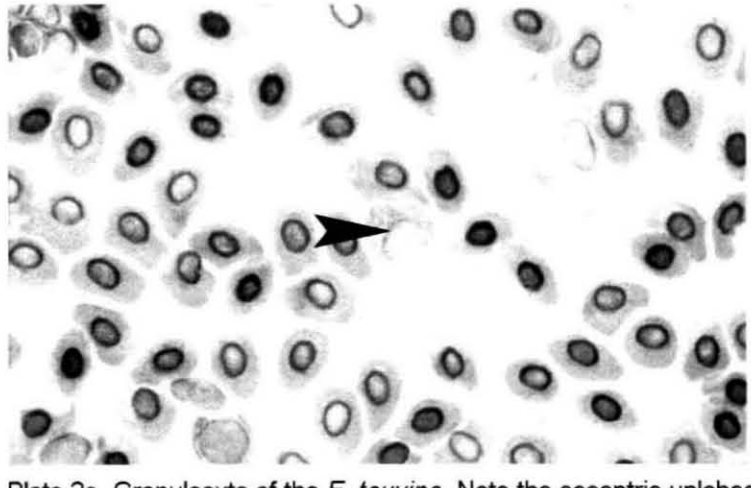


Plate 2c. Granulocyte of the *E. tauvina*. Note the eccentric unlobed nucleus (arrowhead) with the blue granules in the cytoplasm. May-Grunwald and Giemsa stain, 1000 x.

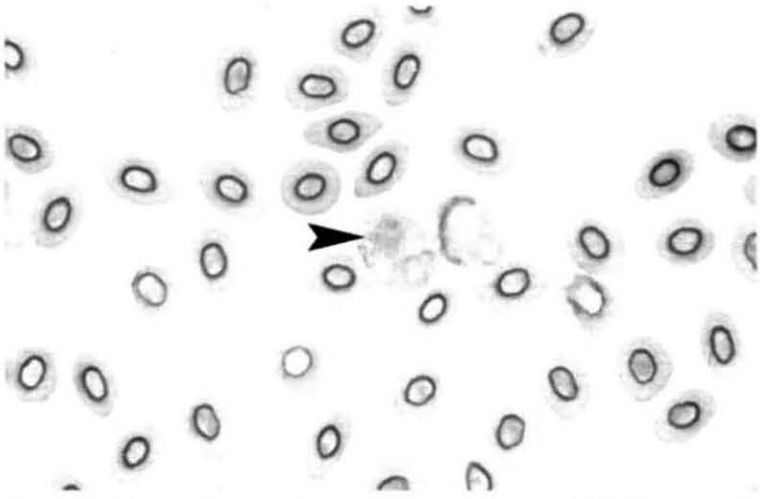


Plate 2d. Neutrophil granulocyte of the *E. tauvina*. Note the centrally placed bilobed nucleus (arrowhead) with blue granules in the cytoplasm. May-Grunwald and Giemsa stain, 1000 x.

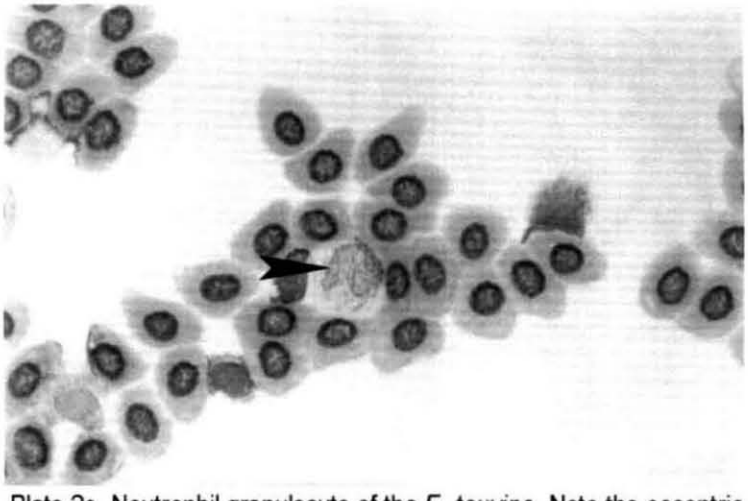


Plate 2e. Neutrophil granulocyte of the *E. tauvina*. Note the eccentric lobed nucleus (arrowhead) with granules in the cytoplasm. May-Grunwald and Giemsa stain, 1000 x.

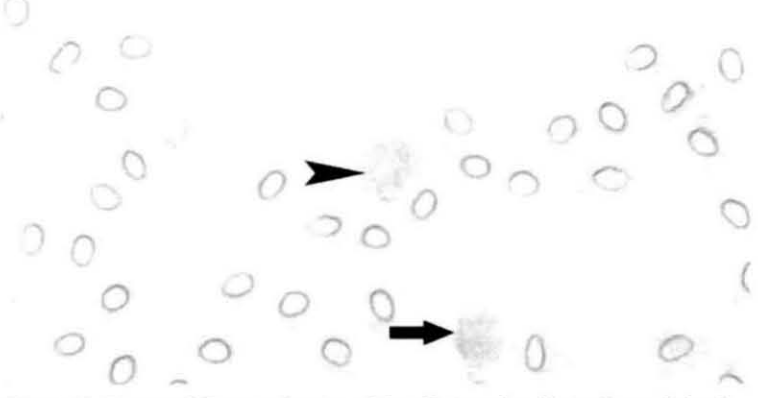


Plate 2f. Neutrophil granulocyte of the *E. tauvina*.Note the unlobed central nucleus (arrowhead) with blue granules in the cytoplasm. A lymphocyte (arrow) is also seen. May-Grunwald and Giemsa stain, 1000 x.

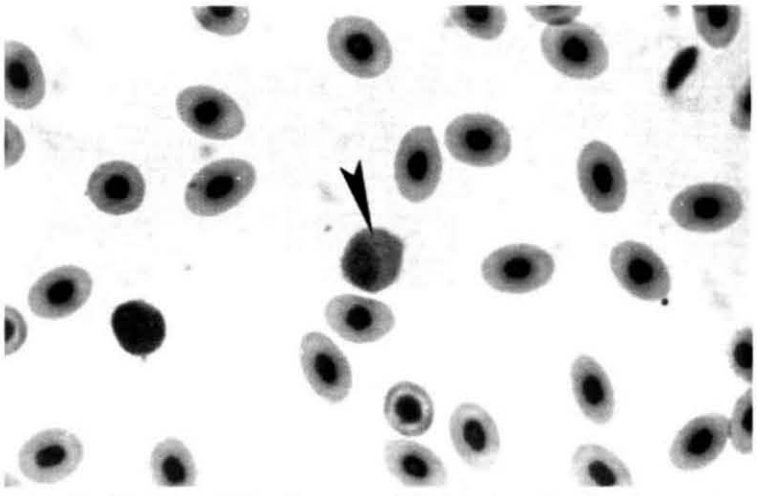


Plate 3a. Peripheral blood smear of *E. tauvina* showing leucocytes with peroxidase positive reaction. Note the blue granules (arrow) in the cytoplasm. Peroxidase stain, 1000 x.

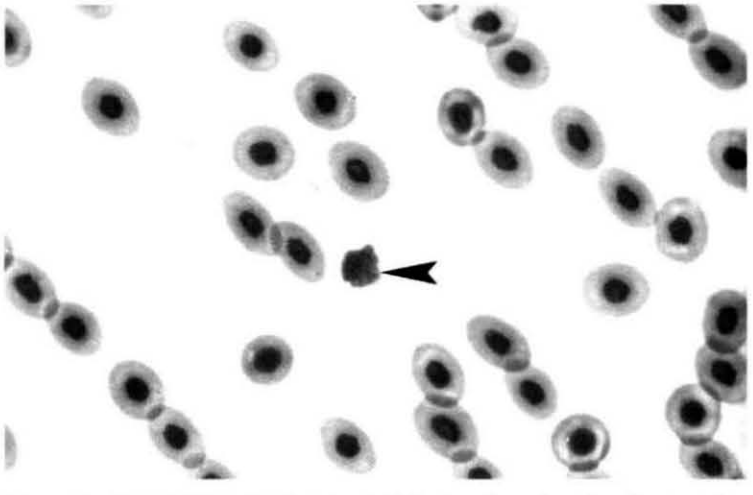


Plate 3b. Peripheral blood smear of *E. tauvina* showing leucocytes with peroxidase positive reaction. Note the blue granules (arrow) in the cytoplasm. Peroxidase stain, 1000 x.

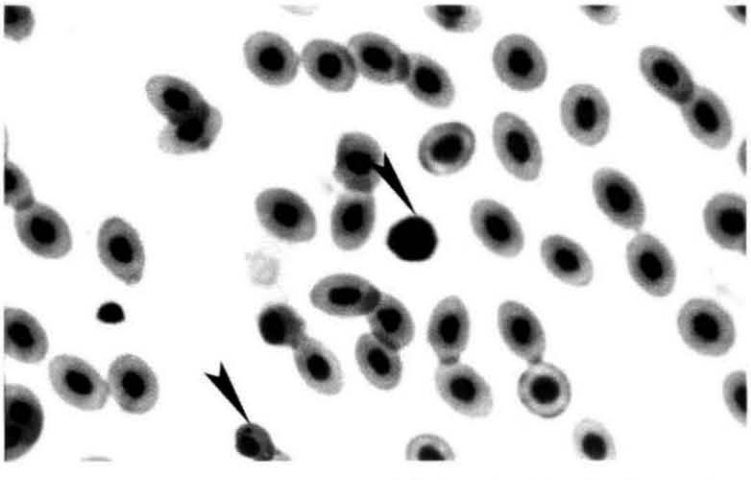


Plate 3c. Peripheral blood smear of *E. tauvina* showing leucocytes with peroxidase positive reaction. Note the blue granules (arrows) in the cytoplasm. Peroxidase stain, 1000 x.

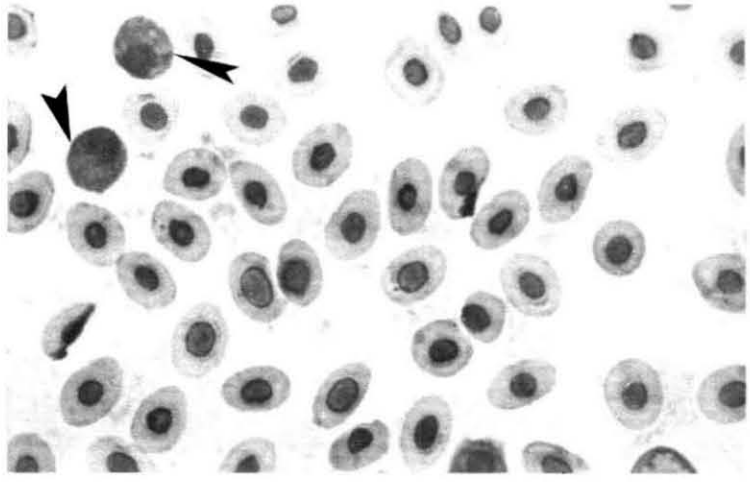


Plate 3d. Peripheral blood smear of *E. tauvina* showing leucocytes with sudan black positive reaction. Note the blue granules (arrowheads) in the cytoplasm. Sudan black B stain, 1000 x.

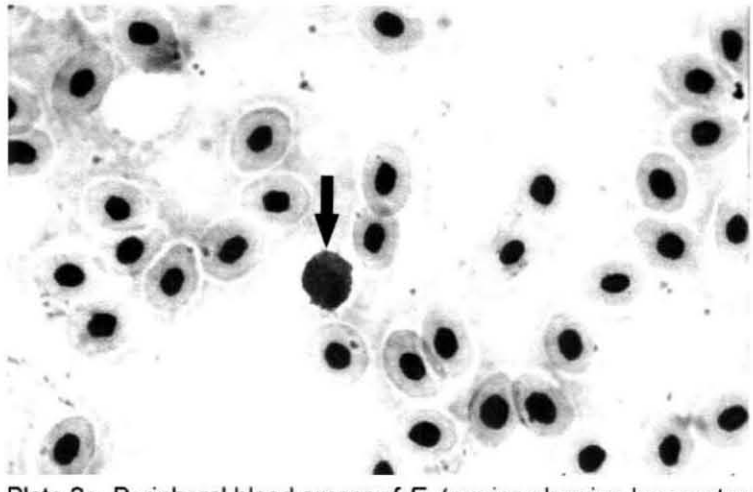


Plate 3e. Peripheral blood smear of *E. tauvina* showing leucocytes with sudan black B positive reaction. Note the eccentric large nucleus with sudan positive granules (arrow) in the cytoplasm. Sudan black B stain, 1000 x.

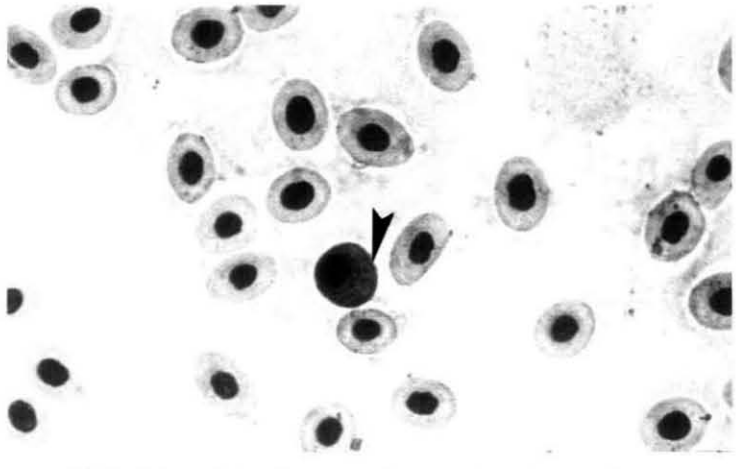


Plate 3f. Peripheral blood smear of *E. tauvina* showing leucocytes with sudan black B positive reaction. Note the eccentric round nucleus with sudan positive granules (arrowhead) in the cytoplasm. Sudan black B stain, 1000 x.

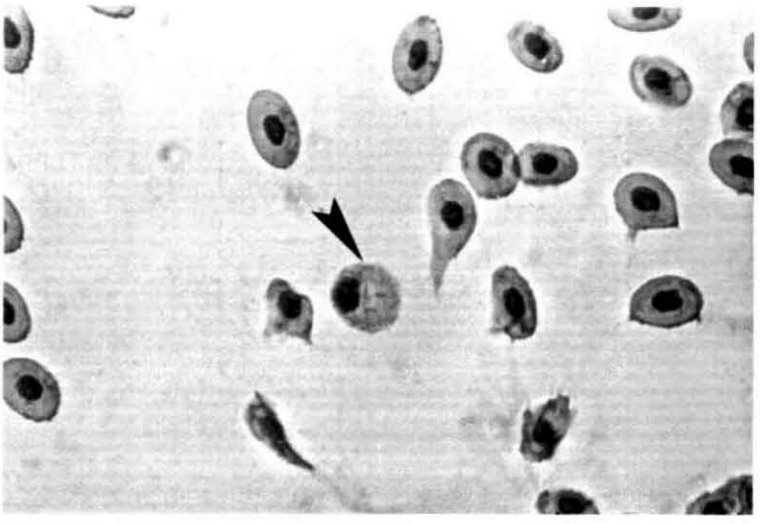


Plate 3g. Peripheral blood smear of *E. tauvina* showing leucocytes with acid phosphatase positive reaction. Note the eccentric round nucleus with granules (arrowhead) in the cytoplasm.
Acid phosphatase stain, 1000 x.

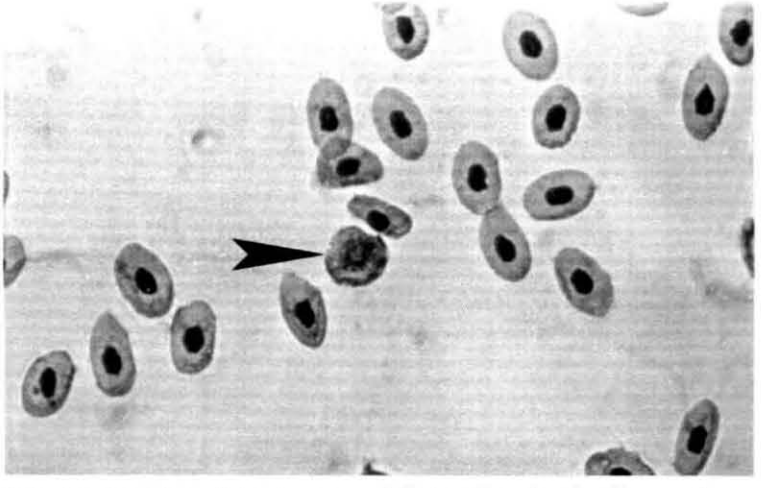


Plate 3h. Peripheral blood smear of *E. tauvina* showing leucocytes with acid phosphatase positive reaction. Note the central nucleus with granules (arrowhead) in the cytoplasm. Acid phosphatase stain, 1000 x.

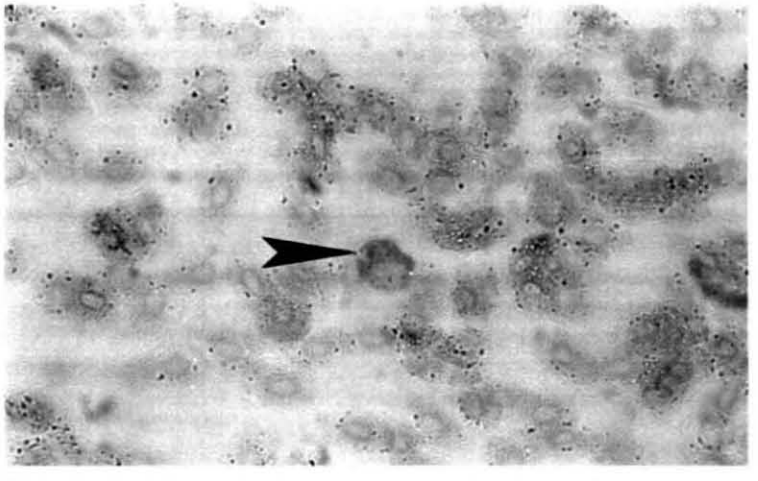


Plate 3i. Peripheral blood smear of *E. tauvina* showing leucocytes with periodic acid Schiff (PAS) positive reaction (arrowhead).
Periodic acid Schiff stain, 1000 x.

developed. Chromatins were located in the periphery of the nuclear membrane of the cells. (Plates 4a - 4g).In the second type, granules had a peripheral electron dense area and a vacuolated central portion. Occasionally these vacuolated areas contained fibrous material or crystalloid inclusions. A few mitochondria were also seen in the cytoplasm. The nuclear chromatin appeared condensed on the periphery of the nucleus.

4.4.2 Monocytes

Monocytes were very few in blood smears and their percentage varied between 3 and 11 and the mean was 6.33 ±1.30. They had a large eccentrically placed nucleus with a foamy or vacuolated cytoplasm. Occasionally a few granules were observed in the cytoplasm. The cytoplasm was mildly positive with PAS and acid phosphatase. (Plates 5a - 5c).

4.4.2.1 Ultrastructure

Monocytes had abundant cytoplasm with a number of mitochondria and vacuoles. Some of the cells had Golgi apparatus and a few stacks of rough endoplasmic reticulum. Cytoplasm also contained lysosomes. (Plates 6a - 6d).

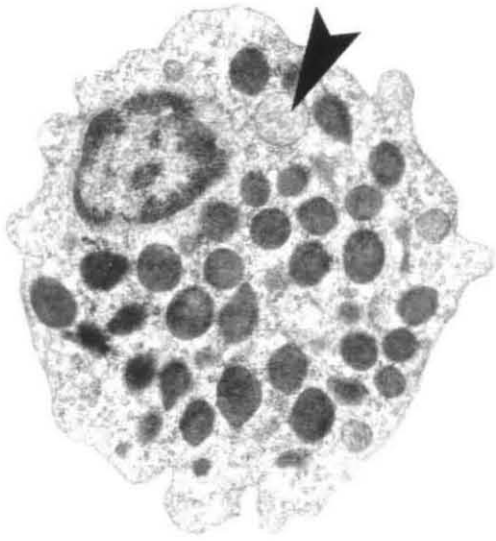


Plate 4a. Electron micrograph of peripheral blood granular leucocyte of *E. tauvina*. Note homogenous electron dense cytoplasmic granules and a few mitochondria (arrowhead). (10000 X).

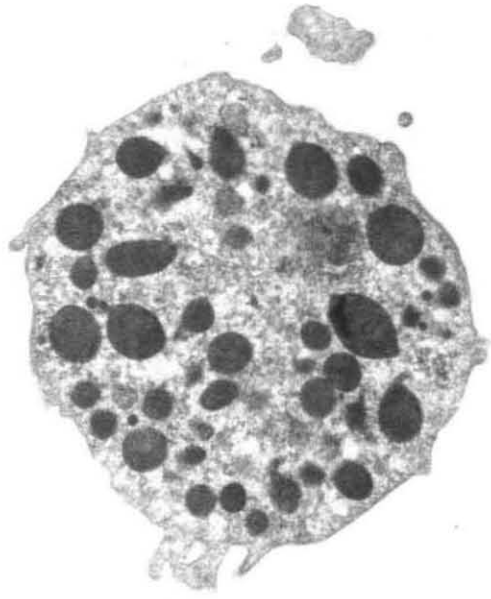


Plate 4b. Electron micrograph of peripheral blood granular leucocyte of *E. tauvina*. Note homogenous electron dense cytoplasmic granules. (4000 X).

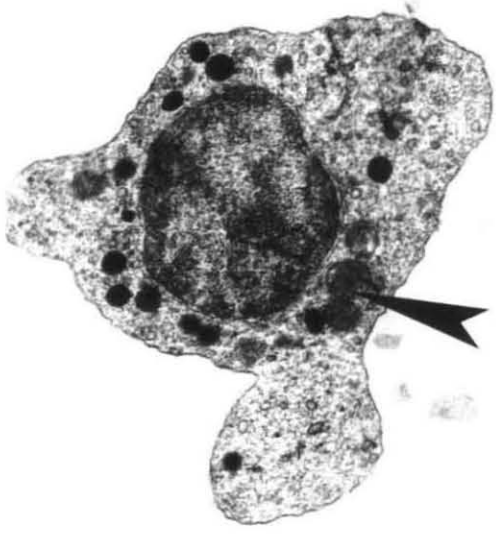


Plate 4c. Electron micrograph of peripheral blood granular leucocyte of *E. tauvina*. Note homogenous electron dense cytoplasmic granules and a few mitochondria (arrowhead). (12000 X).

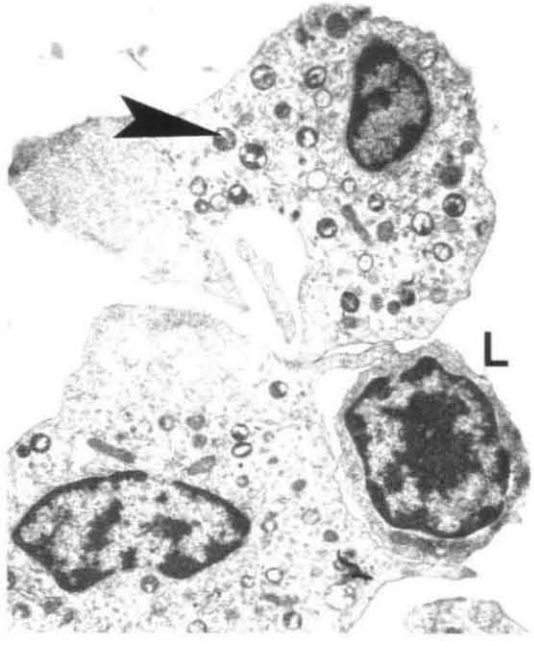


Plate 4d. Electron micrograph of peripheral blood granular leucocytes of *E. tauvina*.The granules containing crystalloid materials (arrowhead) are seen. Note a adjacent lymphocyte (L). (3500X).

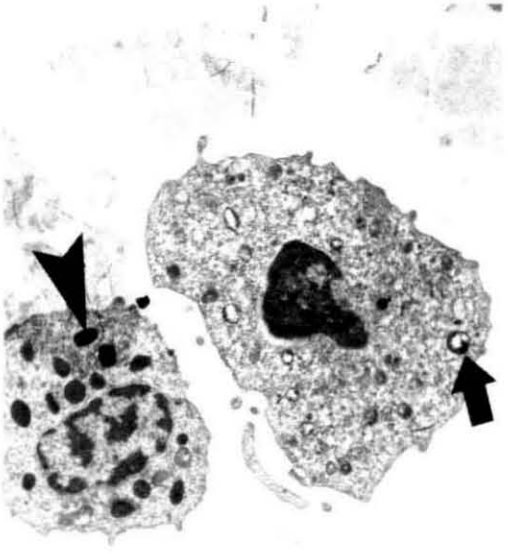


Plate 4e. Electron micrograph of peripheral blood granular leucocytes of *E. tauvina*. Note homogenous electron dense cytoplasmic granules (arrowhead) and crystalloid granules (arrow). (10000 X).

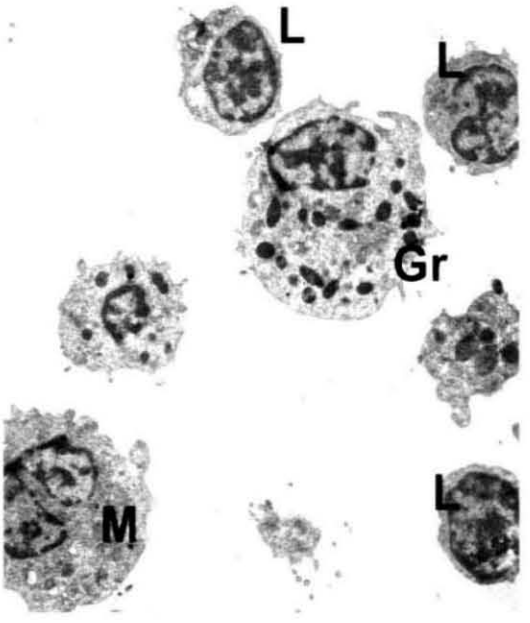


Plate 4f. Electron micrograph showing granulocyte (Gr), lymphocytes (L) and monocyte (M) of *E. tauvina*. Note homogenous electron dense cytoplasmic granules in granulocyte. (8000 X).

Plate 4g. Electron micrograph showing peripheral blood monocytes (M) and lymphocytes (L) of *E. tauvina*. (8000 X).

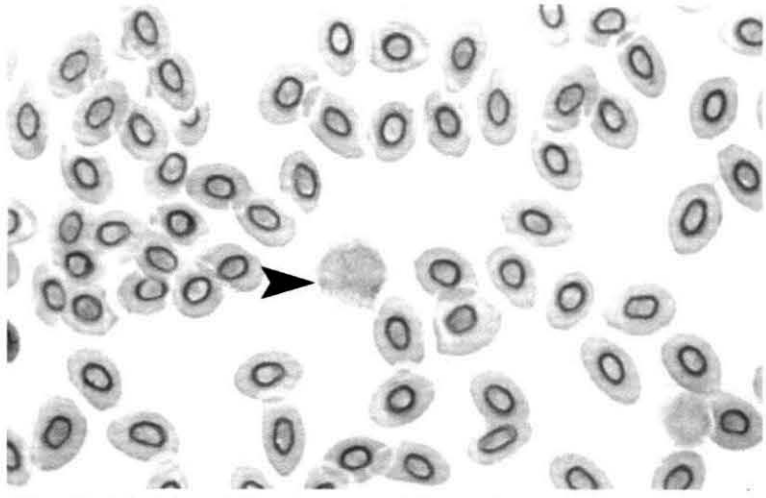


Plate 5a. Peripheral blood smear of *E. tauvina* showing monocyte. Note the large nucleus with foamy cytoplasm (arrowhead) with few granules in the cytoplasm. May-Grunwald & Giemsa stain, 1000 x.

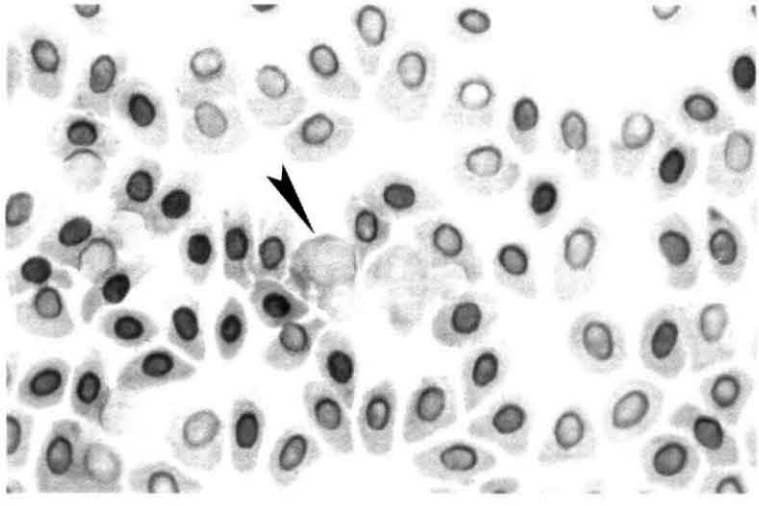


Plate 5b. Peripheral blood smear of *E. tauvina* showing monocyte. Note the large nucleus with foamy cytoplasm (arrowhead) with few granules in the cytoplasm. May-Grunwald & Giemsa stain, 1000 x.

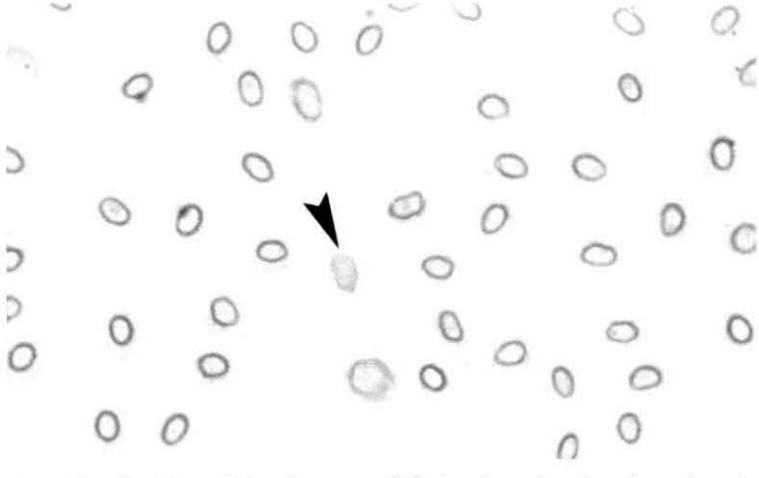


Plate 5c. Peripheral blood smear of *E. tauvina* showing thrombocyte (arrowhead). May-Grunwald & Giemsa stain, 1000 x.

Plate 6a. Electron micrograph of peripheral blood monocyte of *E. tauvina*. Note extended pseudopodia and vacuoles in the cytoplasm. (6000 x).

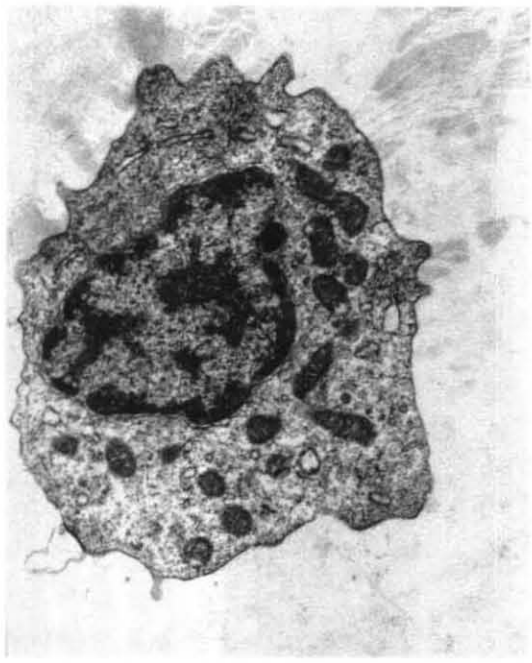


Plate 6b. Electron micrograph of peripheral blood monocyte of *E. tauvina*. Note numerous mitochondria with extended pseudopodia and vacuoles in the cytoplasm . (8000 x).

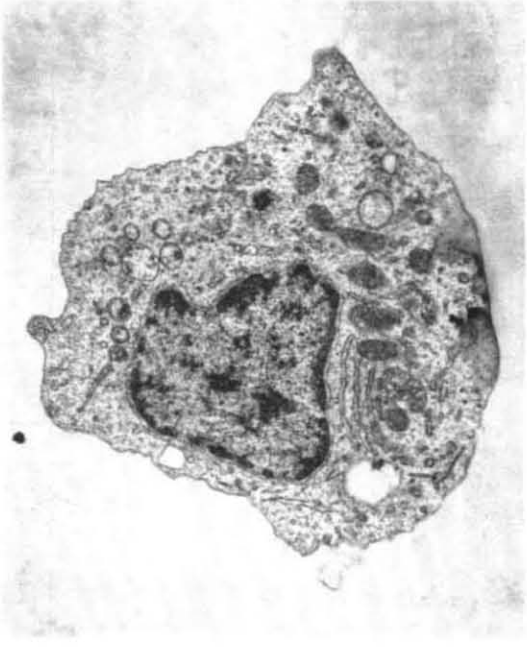


Plate 6c. Electron micrograph of peripheral blood monocyte of *E. tauvina*. Note numerous mitochondria, rough endoplasmic reticulum and vacuoles. (4000 x).

Plate 6d. Electron micrograph of peripheral blood monocyte of *E. tauvina*. Note extended pseudopodia and numerous vacuoles with deposits inside. (10000 x).

4.4.3 Lymphocytes

The numbers of lymphocytes were more in peripheral blood and ranged from 54% to 70%.The mean percentage of lymphocytes was 61.83 ± 2.67. Two types of cells were observed. In one type, cell had large nucleus with peripheral cytoplasm. The cytoplasm appeared slightly basophilic and formed a rim around the nucleus. In other type, the cells appeared small and nucleus occupied the entire cell. (Plates 7a -7c).

4.4.3.1 Ultrastructure

Lymphocytes had very large nucleus and contained very prominent euchromatin and heterochromatin. The cytoplasm contained a few mitochondria and some amount of rough endoplasmic reticulum and abundant polyribosomes. (Plates 8a - 8h).

4.4.4 Thrombocytes

They are elongated, spindle shaped cells. One pole of the cell cytoplasm was thrown out into a point.

4.4.4.1 Ultrastructure

The cytoplasm of thrombocytes contained numerous vacuoles which were interconnected and opened to the exterior through fenestrae. Nucleus was large and consisted of both euchromatin and heterochromatin. (Plates 9a – 9b).

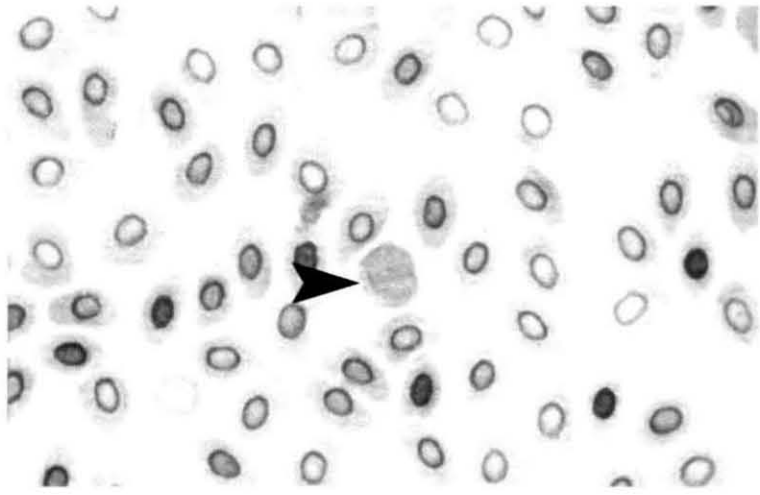


Plate 7a. Peripheral blood smear of *E. tauvina* showing lymphocyte. Note the large nucleus covering entire cytoplasm (arrowhead). May-Grunwald & Giemsa stain, 1000 x.

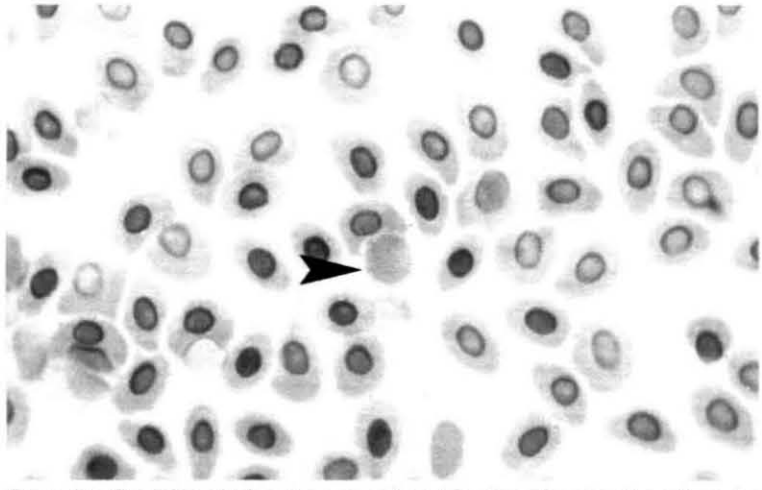


Plate 7b. Peripheral blood smear of *E. tauvina* showing lymphocyte. Note the large nucleus covering entire cytoplasm (arrowhead). May-Grunwald & Giemsa stain, 1000 x.

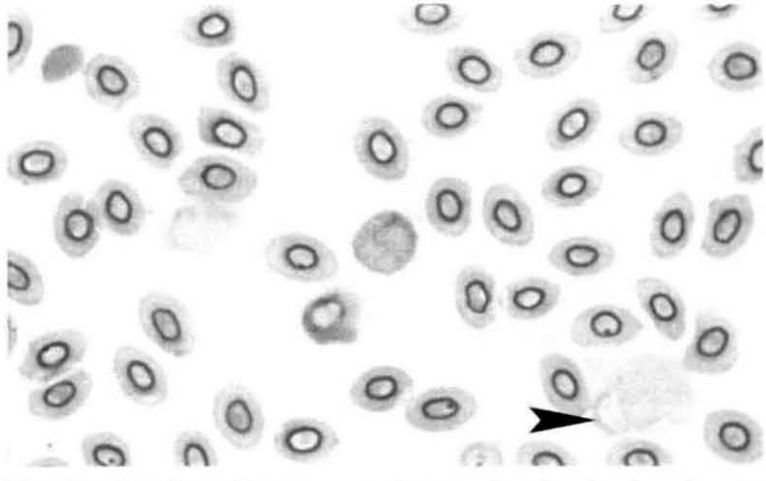


Plate 7c. Peripheral blood smear of *E. tauvina* showing lymphocyte. Note the large nucleus covering entire cytoplasm (arrowhead). May-Grunwald & Giemsa stain, 1000 x.

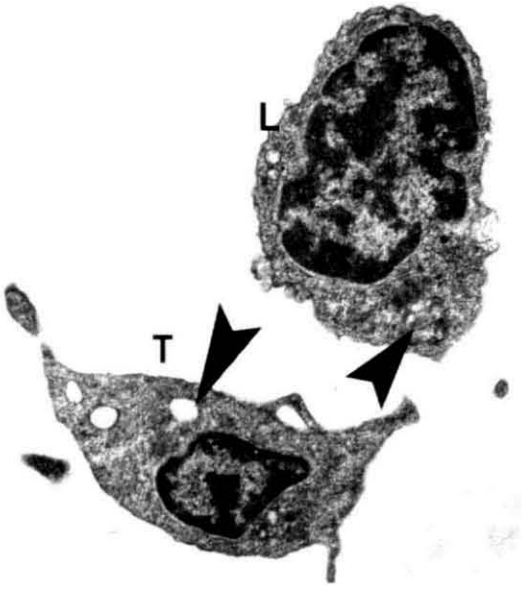


Plate 8a. Electron micrograph of peripheral blood lymphocyte (L) of *E. tauvina*. A thrombocyte (T) is also seen. Note vacuoles in thrombocyte and mitochondria in lymphocytes (arrowheads). (5000 x).

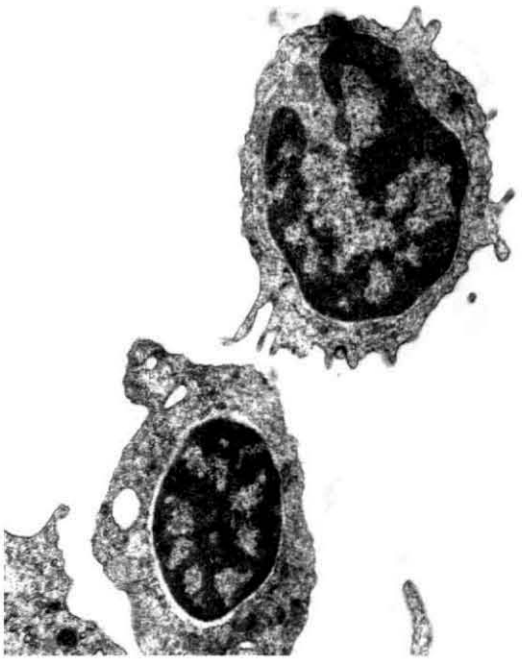


Plate 8b. Electron micrograph of peripheral blood lymphocytes of *E. tauvina*. Note lobed nucleus and extended pseudopodia. (5000 x).

Plate 8c. Electron micrograph of peripheral blood lymphocytes of *E. tauvina*. Note various shape of nucleus with extended cytoplasmic pseudopodia. (5000 x).

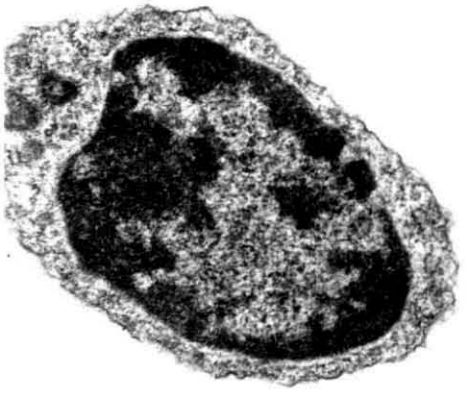


Plate 8d. Electron micrograph of peripheral blood lymphocyte of *E. tauvina*. (8000 x).

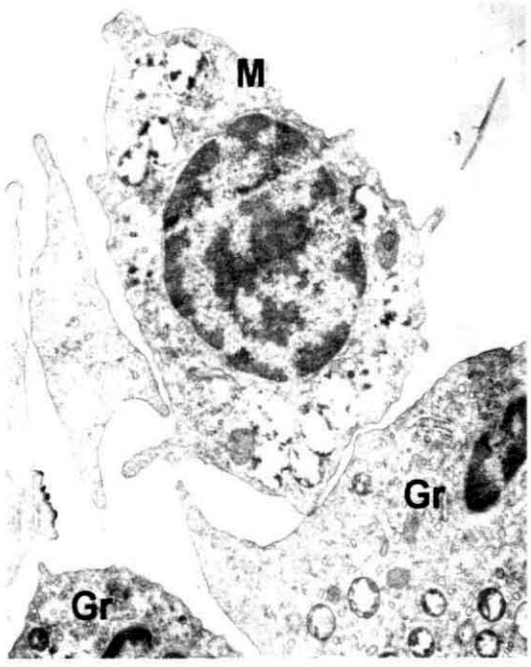


Plate 8e. Electron micrograph of peripheral blood monocyte (M) and granulocytes (Gr) of *E. tauvina*. Note central nucleus with few mitochondria. (5000 X).

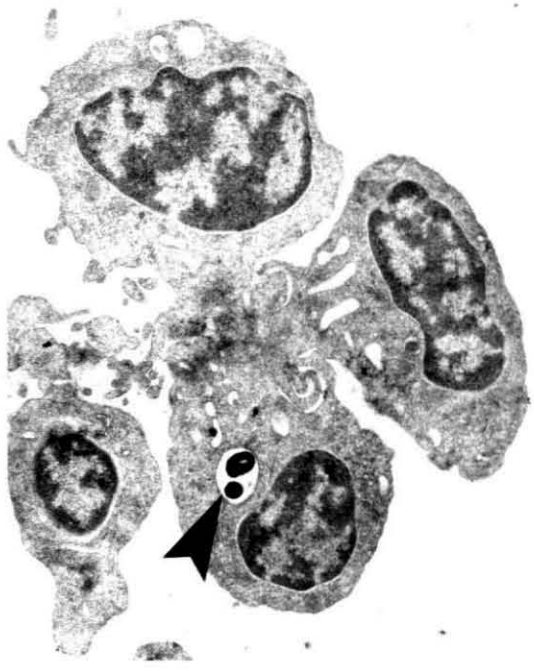


Plate 8f. Electron micrograph of peripheral blood lymphocytes of *E. tauvina*. Note a vacuole (arrow head) deposited with phagocytic material. (8000 X).

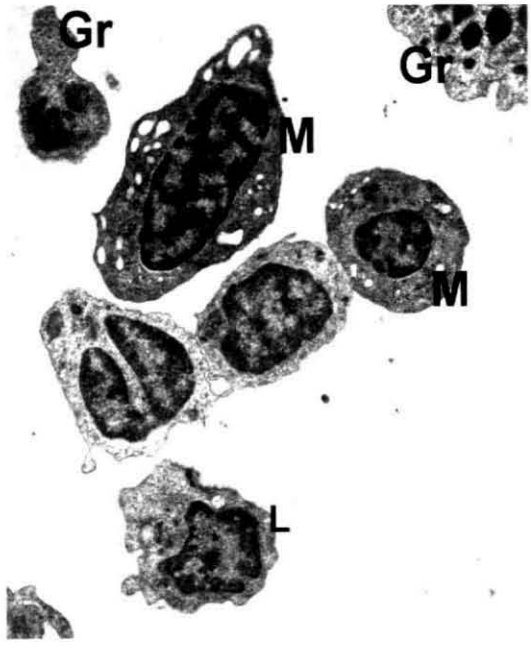


Plate 8g. Electron micrograph of peripheral blood leucocytes of *E. tauvina*. Note granulocytes (Gr), monocytes (M) and lymphocytes (L). (10000 X).

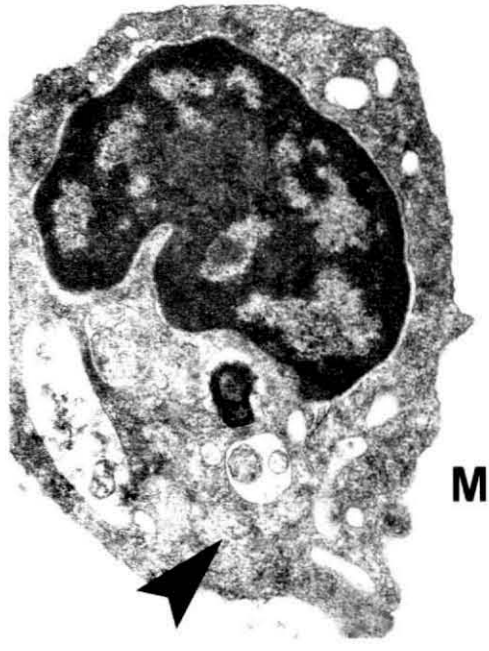


Plate 8h. Electron micrograph of peripheral blood monocyte of *E. tauvina*. Note eccentric nucleus with mitochondria (arrow head). (3500 X).

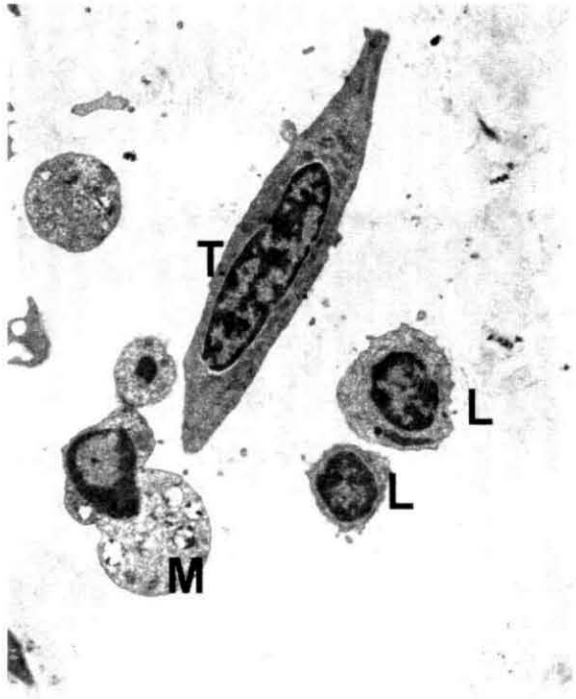


Plate 9a. Electron micrograph of peripheral blood thrombocyte (T) of *E. tauvina*. Note monocyte(M) and lymphocytes (L). (12000 X).

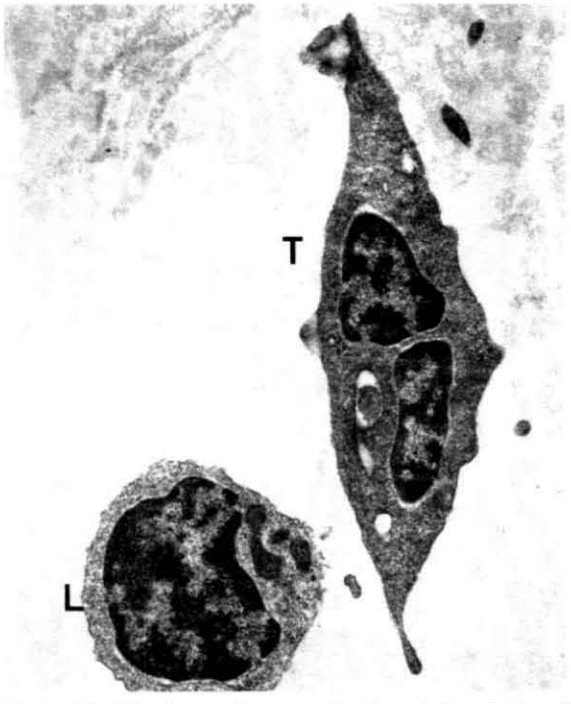


Plate 9b. Electron micrograph of peripheral blood thrombocyte (T) of *E. tauvina*. Note central lobed nucleus. A lymphocyte (L) occupied with nucleus is also seen. (3500X).

4.5 LYMPHOID ORGANS

4.5.1 Thymus

Thymus gland was a well developed, paired organ present in dorsolateral region of the gill chamber in *Epinephelus tauvina*.

The structural organization of the thymus can be described as follows. The entire thymus was surrounded by a connective tissue capsule that projected as several trabeculae into thymic parenchyma. The trabeculae were consisting of fibroblasts, collagen fibres and myo-epithelial cells. The thymic parenchyma also contained numerous lymphocytes, myoid cells and epithelial cells. The differentiation of thymic parenchyma into cortex and medulla was not seen. (Plates 11a – 11b)

The special staining techniques revealed the following information. The capsule and trabeculae contained large amount of collagenous tissue. The trabeculae extended into the parenchyma and it carrired the blood vessels and nerves. The connective tissue of the trabeculae also sent collagen tissue into the matrix. In the parenchyma the main supporting tissue of the matrix was formed by reticular fibres. These reticular fibre networks divided the thymus into small lobules. The lobules contained epithelial cells in close association with thymocytes /lymphoblasts. The epithelial cells formed a covering around the blood capillaries also. In addition to the epithelial cells and lymphoblasts, thymus contained macrophages, myoid cells and myofibrils in lobules. (Plates 12a – 12c and 13a -13b).

4.5.1.1 Ultrastructure

The ultrastructure of thymus showed many blood capillaries which were surrounded by epithelial cells. The capillaries lumen was filled with blood cells. Most of the blood capillaries were lined by endothelial cells with tight junctions. The lumen of capillaries was filled with blood cells. Occasionally some of the epithelial cells were fenestrated in appearance while others had electron dense cytoplasm.

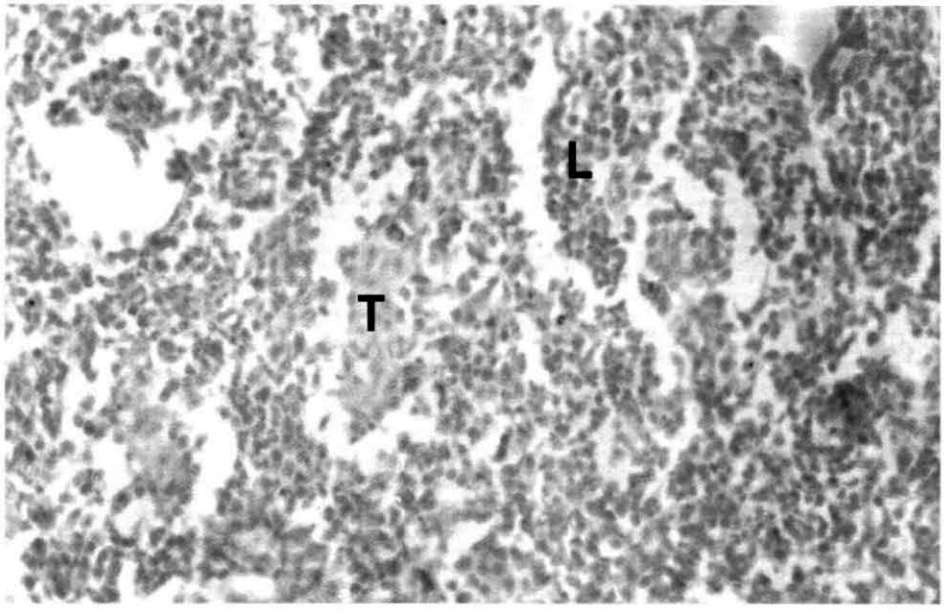


Plate 11a. Section of thymus of *E. tauvina* showing lymphoblasts (L) and portion of trabecula (T). H & E, 400 x.

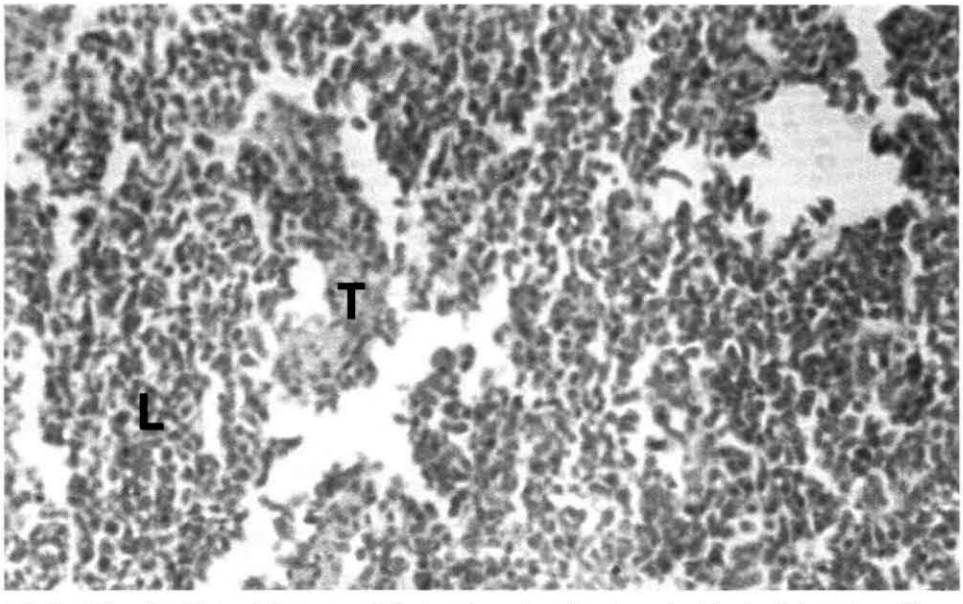


Plate 11b. Section of thymus of *E. tauvina* showing lymphoblasts (L) and portion of trabecula (T). H & E , 400 x.

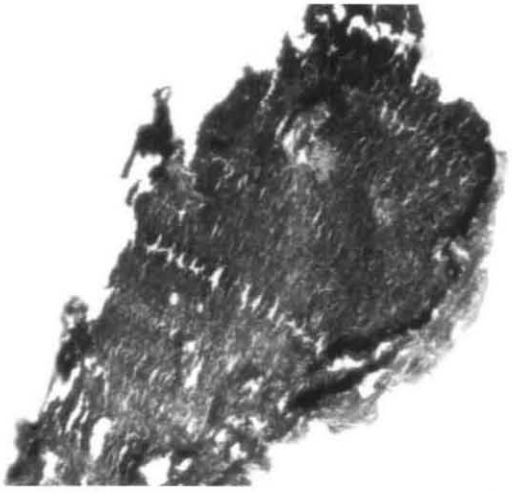


Plate 12a. Section of thymus of *E. tauvina* showing the distribution of collagen tissue which has taken blue colouration. A thick blue area indicates trabecula, red area mostly consisting of lymphoid cells and epithelial cells. Trichrome - Masson stain, 100 x.

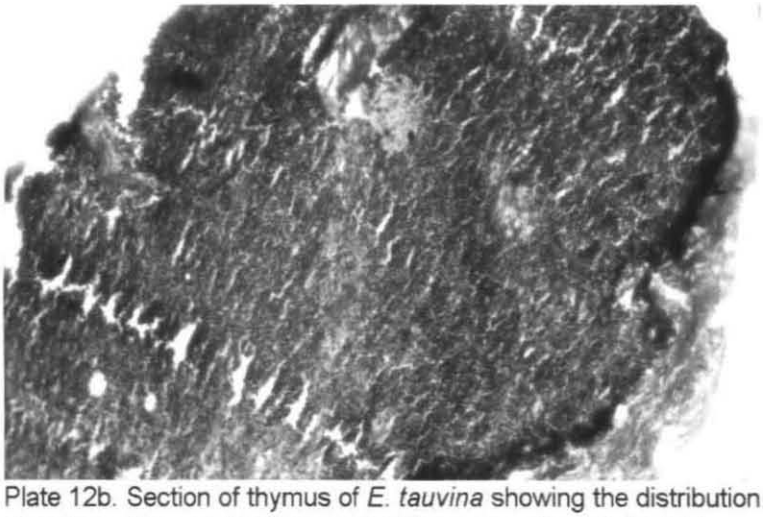


Plate 12b. Section of thymus of *E. tauvina* showing the distribution of collagen tissue which has taken blue colouration. A thick blue area indicates trabecula, red area mostly consisting of lymphoid cells and epithelial cells. Trichrome - Masson stain, 200 x.

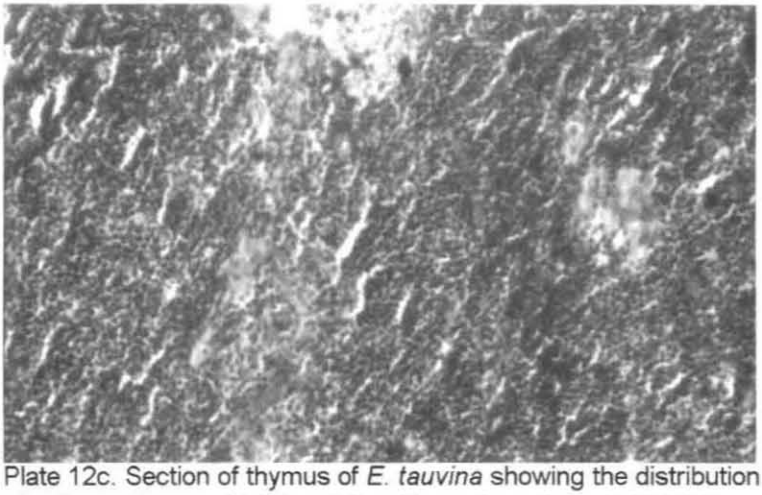


Plate 12c. Section of thymus of *E. tauvina* showing the distribution of collagen tissue which has taken blue colouration. A thick blue area indicates trabecula, red area mostly consisting of lymphoid cells and epithelial cells. Trichrome - Masson stain, 400 x.

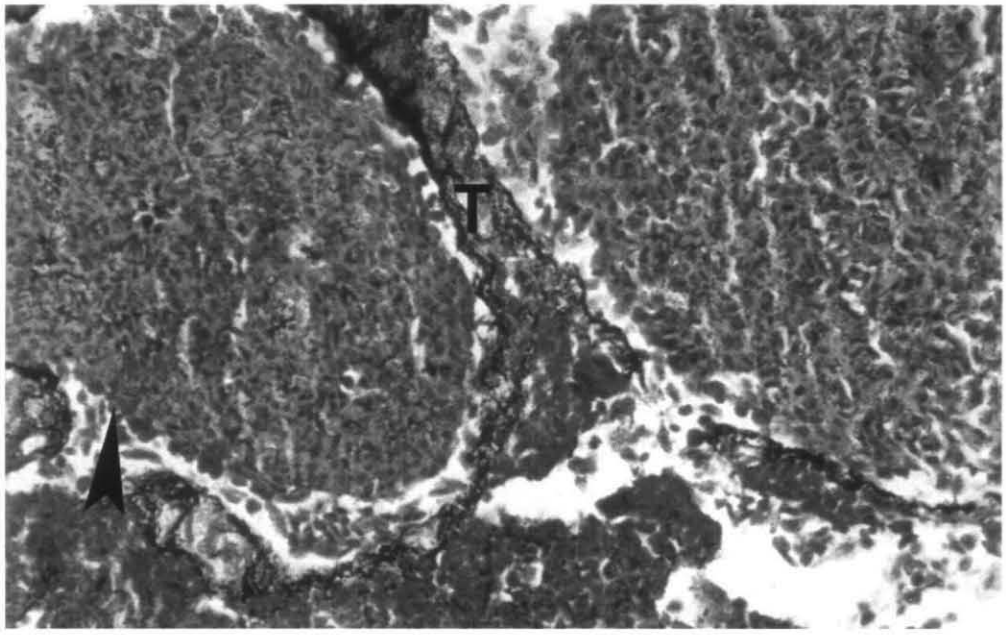


Plate 13a. Section of thymus of *E. tauvina*. Note the arrangement of lobules (arrowhead) and trabecula (T). Reticulum - Gomori stain, 400 x.

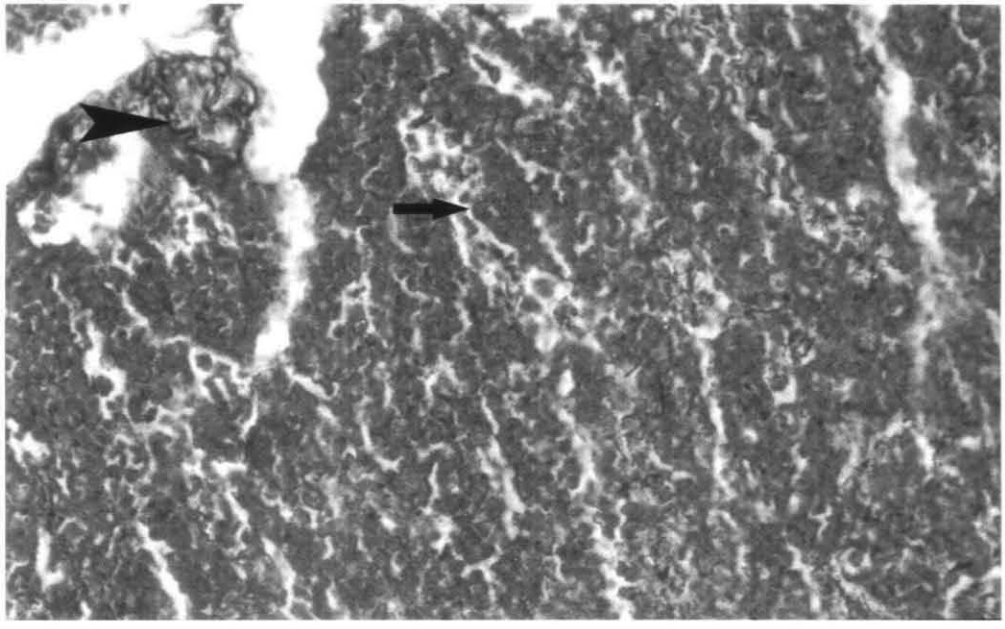


Plate 13b. Section of thymus of *E. tauvina* depicting the distribution of stromal reticular connective tissue (arrowhead) .Note the arrangement of lymphoid tissue into lobules (arrow). Reticulum- Gomori stain, 400x.

In association with epithelial cells and also in the lumen of capillaries numerous developing lymphoblasts were seen. (Plates 14a – 14h).

4.5.2 Kidney

Kidneys of the *Epinephelus tauvina* are capsulated, dark-red organs extending ventrally along the vertebral column. The kidneys are paired organs and each kidney is separated into anterior and posterior regions.

The anterior kidney consisted of exclusively haemopoietic tissues supported by reticular connective tissue while the posterior kidney was formed nephrons surrounded by haemopoietic tissue and stromal tissue. The anterior kidney was mainly for haemopoietic activities whereas posterior kidney showed renal and endocrinal tissue. Numerous blast cells in stromal tissue were observed in the posterior kidney. Further, numerous blood sinuses lined with endothelial cells were also observed in both the anterior and posterior regions of the kidney. The developing leucocytes were observed within and outside the sinuses, scattered in random throughout the stroma. Melanomacrophage centers were also found among the haemopoietic tissue. They are nodular and pale in colour. Such centres were located near the vascular channels and had lymphocytes inside. (Plates 15a – 15e).

4.5.2.1 Ultrastructure

The fine structure of kidney revealed the presence of numerous lymphoblasts and granulocytes in the cellular matrix. Some of the granulocytes observed were containing crystalloid granules while others had dense cytoplasmic granules. The parenchymal tissues contained fibroblasts, mesenchymal cells and numerous blood sinuses. (Plates 16a -16f).

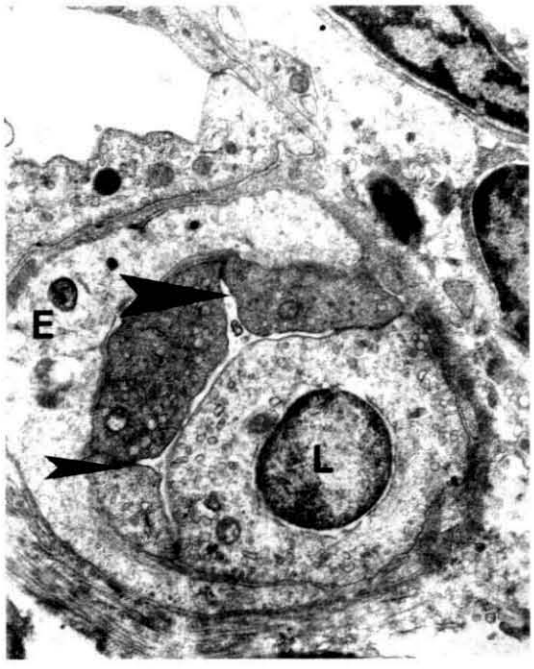


Plate14a. Section of thymus of *E. tauvina* showing a blood capillary with tight endothelial junctions(arrowheads) and developing lymphoblasts(L). Note epithelial cells (E) covering blood vessels with tight junctions. (5000 X).

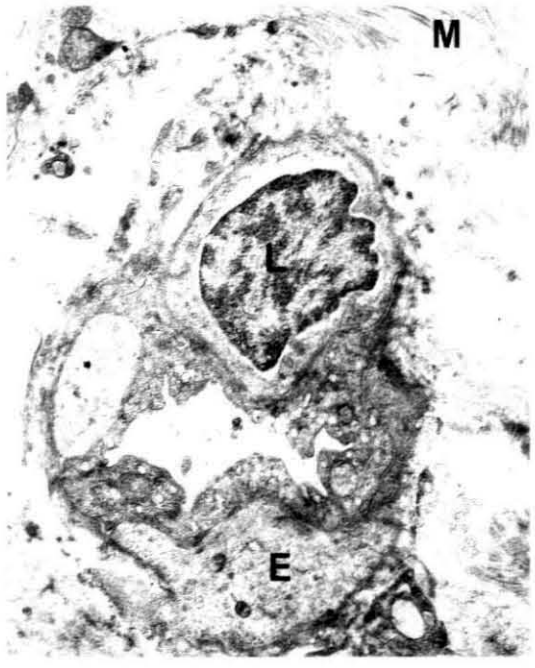


Plate 14b. Section of thymus of *E. tauvina* showing lymphoblasts (L), portion of epithelial cells(E), reticular cells and myofibrils (M). (3500 X).

Plate 14c. Section of thymus of *E. tauvina* showing portion of epithelial cells(E) covering blood vessels. Note a lymphoid cell (L) inside the lumen of blood vessel.(3500 X).

Plate 14d. Section of thymus of *E. tauvina* showing developing lymphoblasts (L)inside blood sinus. (3500 X).

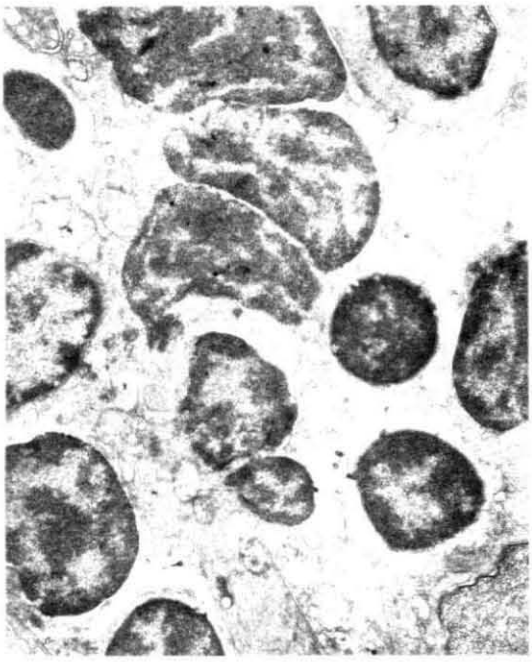


Plate 14e. Electron micrograph of thymus of *E. tauvina* showing lymphoid cells at different stages of development.(3500 X).

Plate 14f. Electron micrograph of thymus of *E. tauvina* showing developing lymphoblasts(L) in association with epithelial cell (E).(3500 X).

Plate 14g. Electron micrograph of thymus of *E. tauvina* showing epithelial cells (E) and lymphoid cells (L). (3500 X).

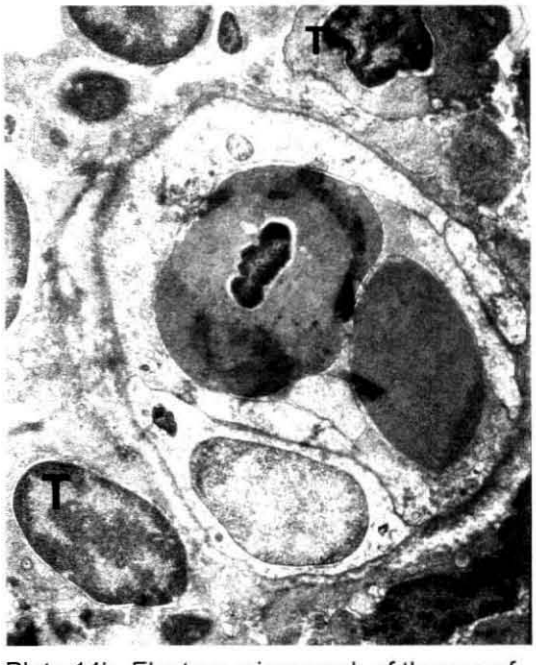


Plate 14h. Electron micrograph of thymus of *E. tauvina* showing blood capillaries and developing thymocytes (T). (3,500 X).

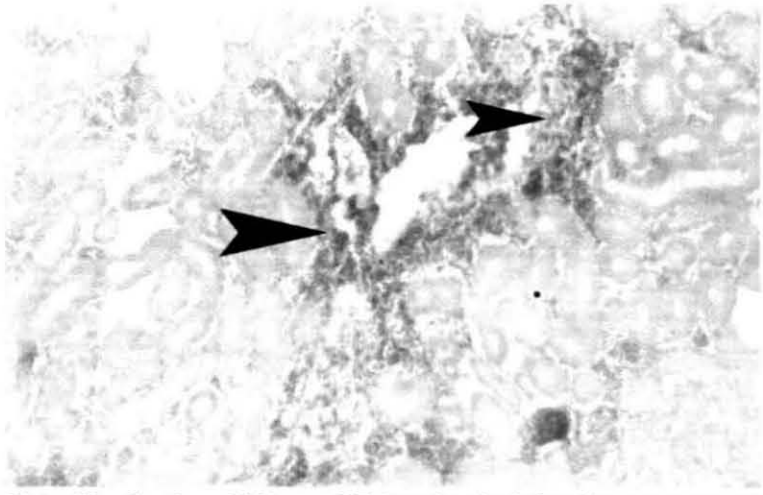


Plate 15a. Section of kidney of *E. tauvina* depicting the arrangement of nephrons and intertubular distribution of haemopoietic tissue (arrowheads). H & E, 200 x.

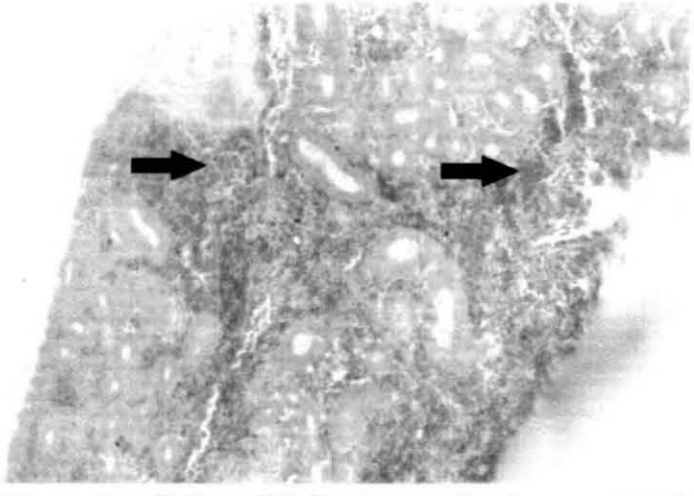


Plate 15b. Section of kidney of *E. tauvina* revealing the arrangement of nephrons and intertubular distribution of haemopoietic tissue (arrows). H & E, 200 x.

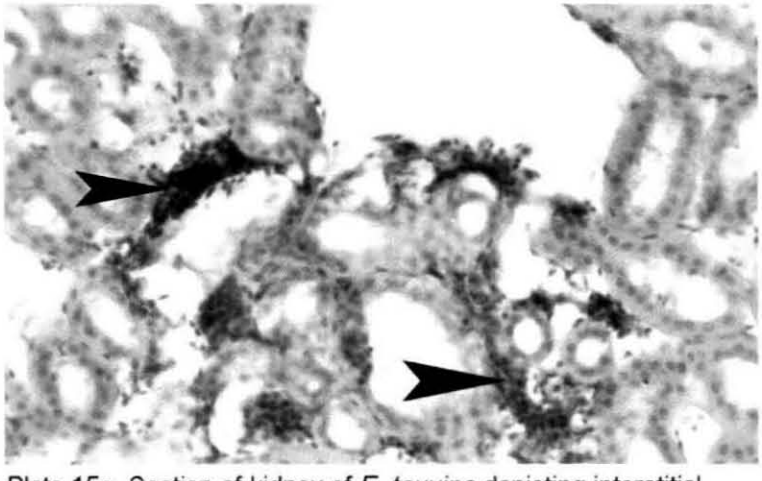


Plate 15c. Section of kidney of *E. tauvina* depicting interstitial distribution of haemopoietic tissue (arrowheads). H & E, 400 x.

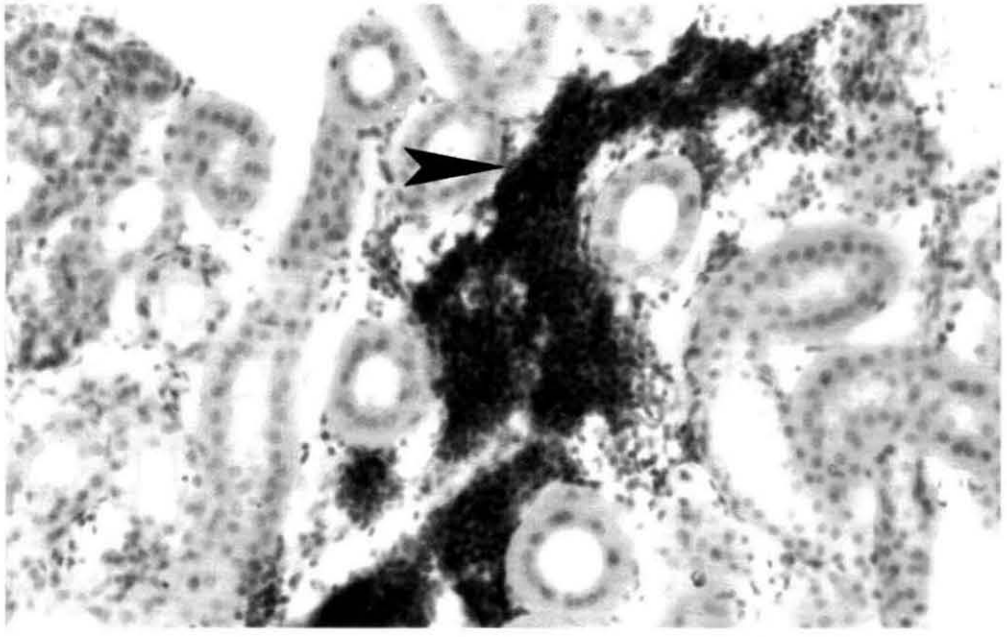


Plate 15d. Section of kidney of *E. tauvina* showing haemopoietic tissue inbetween tubules of nephrons (arrow). H & E, 400 x.

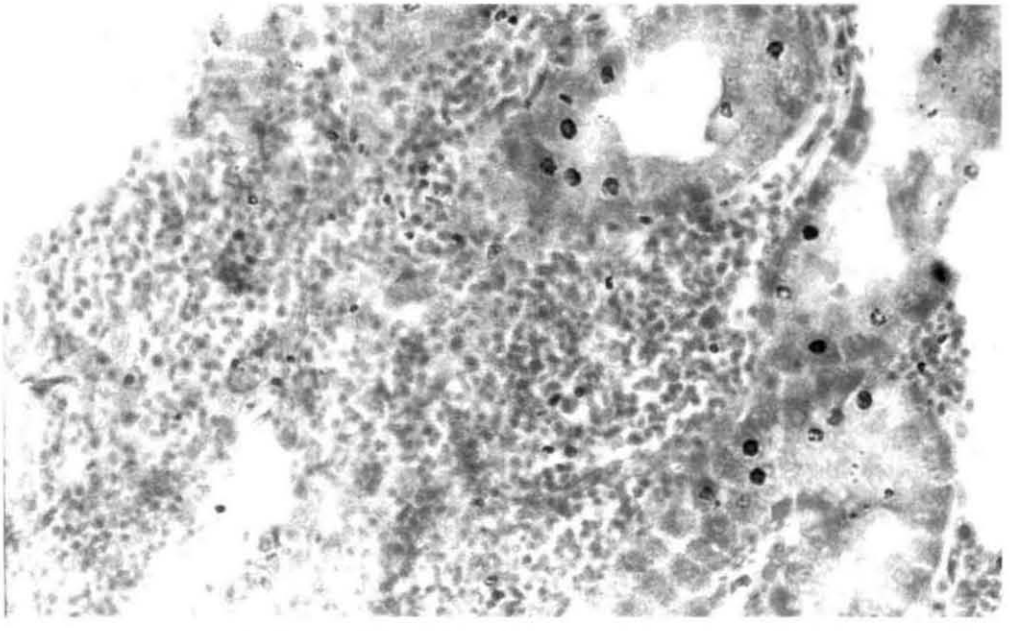


Plate 15e. Section of kidney of *E. tauvina* showing extensive area of haemopoietic tissue in the anterior portion of the kidney. Verhoeff's Van Gieson stain, 400 x.

Plate 16a. Electron micrograph of kidney of *E. tauvina* depicting the portion of blood vessels and various lymphoid cells. (3500 X).

Plate 16b. Electron micrograph of kidney of *E. tauvina* depicting various types of lymphocytes (L) and developing blast cells.(4000 x).

Plate 16c. Electron micrograph of kidney *E. tauvina* depicting neutrophil (N) granulocytes and a lymphoblasts (L).(4000 x).

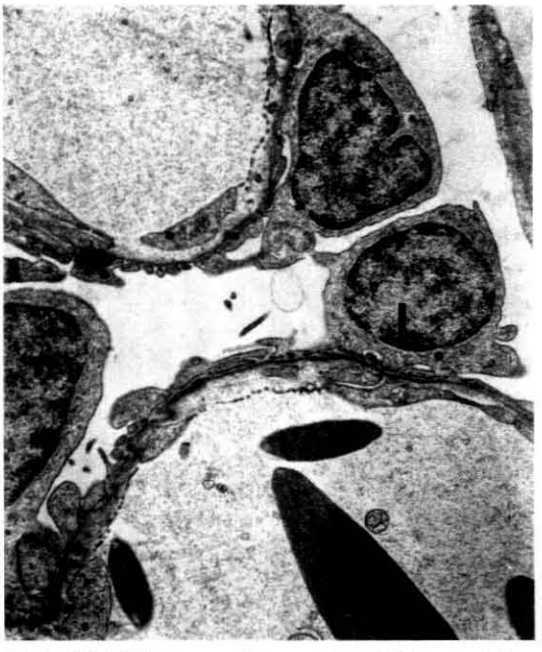


Plate 16d. Electron micrograph of kidney of *E. tauvina* depicting blood capillaries and adhering lymphoid cells (L). (3500 x).

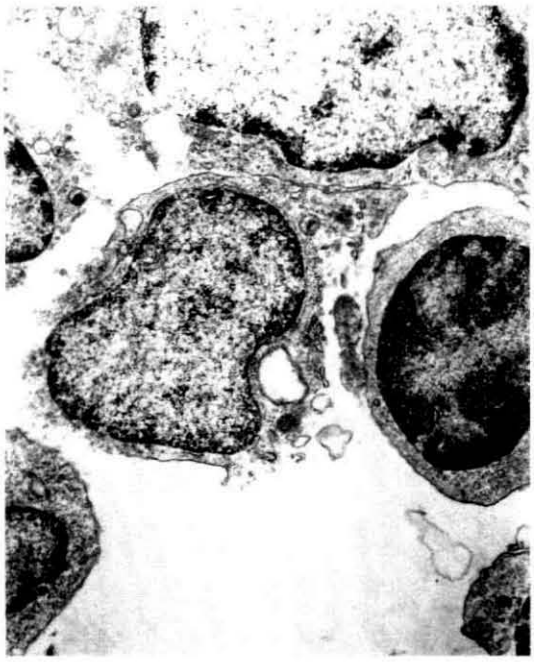


Plate 16e. Electron micrograph of kidney of *E. tauvina* showing various lymphoid cells. (3,500 X).

Plate 16f. Electron micrograph of kidney of *E. tauvina* showing a part of blood vessels and developing leucocytes. (3,500 X).

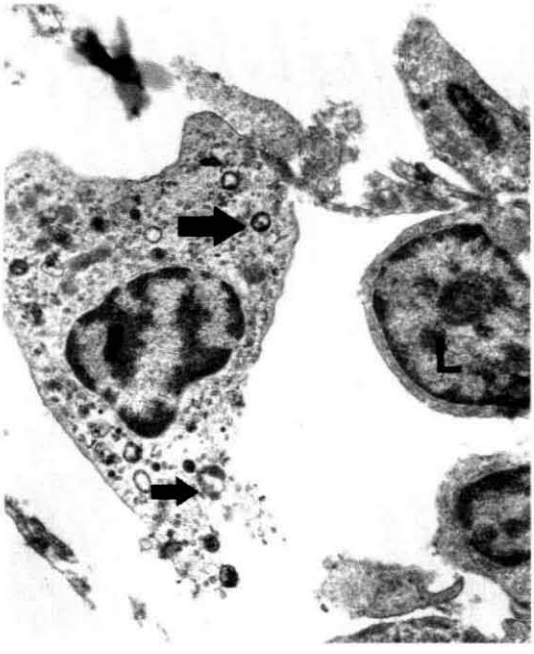


Plate 16g. Electron micrograph of kidney of *E. tauvina*. Note crystalloid and fibrous material inside granules. Lymphocytes (L) are also seen. (3,500 X).

Plate 16h. Electron micrograph of kidney of *E. tauvina* showing developing lymphoid tissue (3,500 X).

4.5.3 Spleen

In *Epinephelus tauvina*, the spleen appears as a dark red, elongated, cylindrical rod like structure, situated very near to the greater curvature of the stomach or flexure of intestine.

The spleen was composed of both white and red pulp but these were not well differentiated. They were scattered through out the spleen. The spleen was surrounded by a collagenous spleenic capsule and such capsule was composed of fibrous connective tissue. Both mesenchymal and parenchymal tissues were randomly distributed in the spleen. The mesenchyma was the supporting network consisting of arteries, veins, capillaries, reticular cells, reticular fibers etc. While parenchyma was composed of numerous blood sinuses and blood cells. The parenchyma contained the lymphoid tissues carrying several types of lymphoid cells. The cells of haemopoietic tissue were distributed on a mesh work formed by the supporting reticular connective tissue. The small capillaries were surrounded by a sheath of reticular connective tissue.

The sheaths of reticular connective tissue surrounding the capillaries were filled with numerous macrophages and lymphoid cells. This was designated as spleenic ellipsoids. Such structures were noticed in *Epinephelus tauvina* spleen. The spleenic ellipsoids opened to spleenic cords, which have blood sinuses. The endothelial lining of ellipsoids and blood sinuses appeared to have numerous gaps or fenestrations. Few pale coloured focal melanomacrophage centers were also observed very near to ellipsoids. (plates 17a – 17c).

The special staining reactions of trichrome Masson, Verhoff's Van Gieson and reticulum Gomori stainings revealed important structural details of spleen. The spleinc ellipsoids were enclosed with reticular and collagen fibre sheath. This reticular collagen fibre sheath around blood vessels formed the stroma for housing macrophages, lymphocytes and also for antigen trapping. Similar network of reticular sheath was also noticed extending to melanomacrophage centres. (Plates 18a -18c).

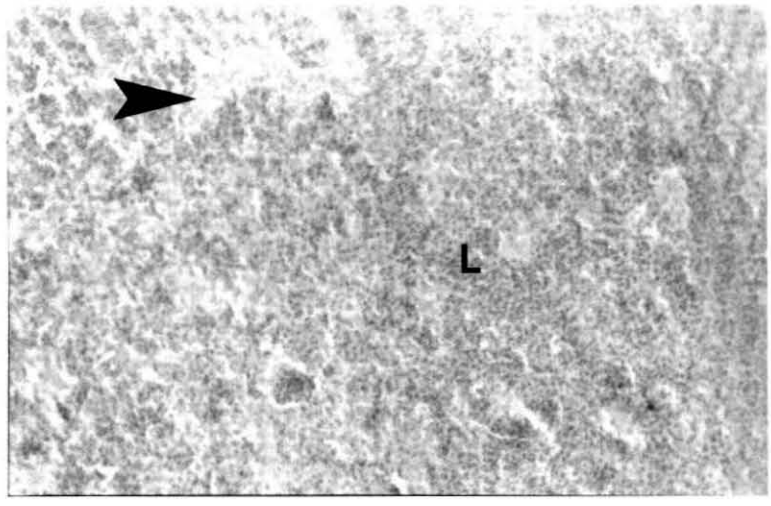


Plate 17a. Section of spleen of *E. tauvina* showing ellipsoids (arrowhead) and lymphoid tissue (L). H & E , 200 x.

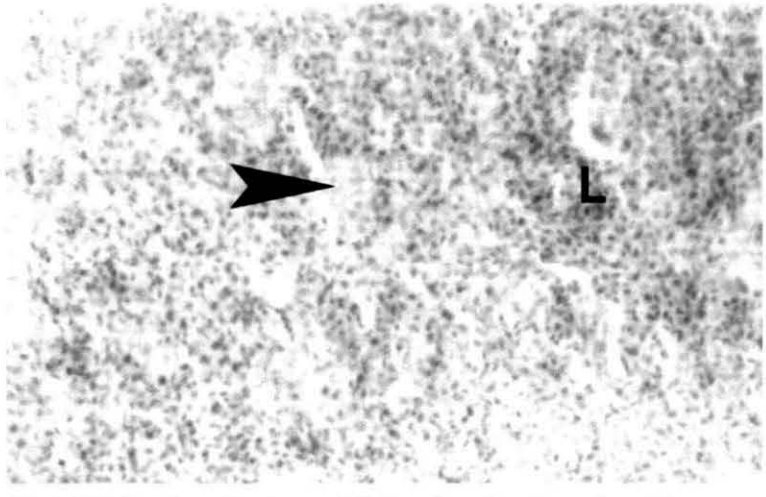


Plate 17b. Section of spleen of *E. tauvina* showing ellipsoids (arrowhead) and lymphoid tissue (L). H & E , 400 x.

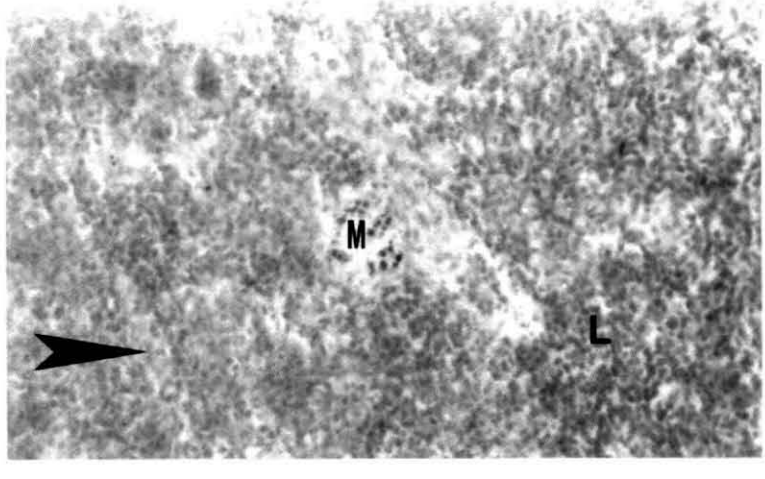


Plate 17c. Section of spleen of *E. tauvina* showing ellipsoids (arrowhead), melanomacrophage center (M) and lymphoid tissue (L). H & E , 400 x.

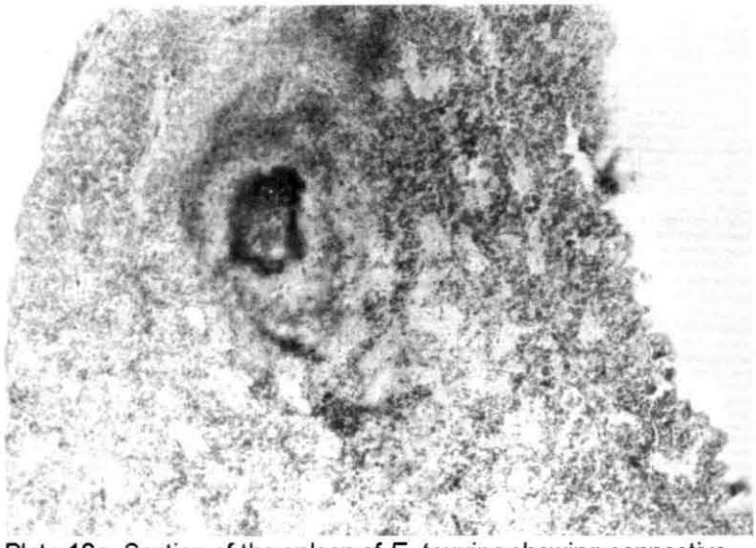


Plate 18a. Section of the spleen of *E. tauvina* showing connective tissue frame work around blood vessels. The collagens stained deep blue around the blood vessels and pale blue around ellipsoids. Trichrome - Masson, 100 x.

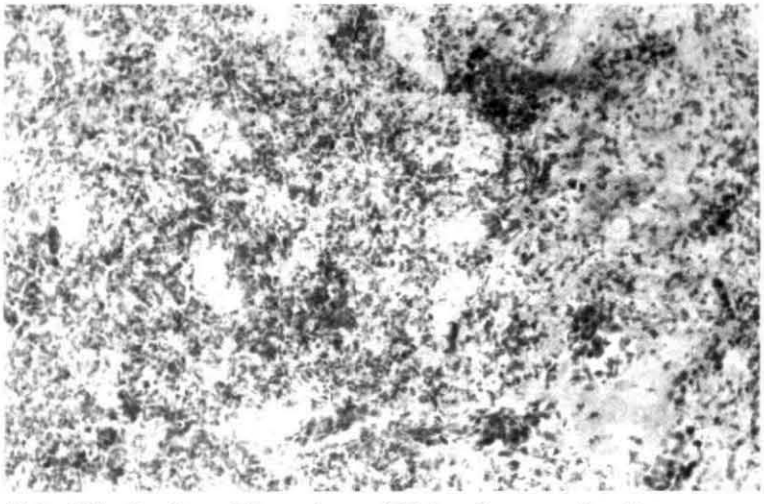


Plate 18b. Section of the spleen of *E. tauvina* revealing the distribution of connective tissue frame work of the spleen. Note the pale blue collagen around ellipsoids. Trichrome - Masson, 200 x

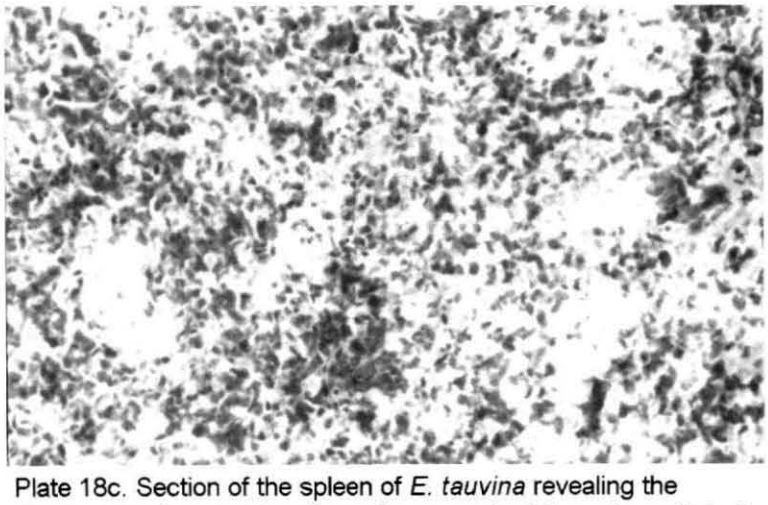


Plate 18c. Section of the spleen of *E. tauvina* revealing the distribution of connective tissue frame work of the spleen. Note the pale blue collagen around ellipsoids. Trichrome - Masson, 400x.

4.5.3.1 Ultrastructure

Transmission electron microscopy of *Epinephelus tauvina* spleen revealed the phenomenon of haemopoiesis in blood sinuses. The blood sinuses contained numerous erythrocytes, granulocytes, lymphocytes and macrophages. These cells were very close to endothelial cells present in the capillaries and blood sinuses. (Plates 19a – 19f).

4.5.4 Gut associated lymphoid tissue

Intestinal mucosa was thrown into several projecting folds which were lined by columnar epithelium having a brush boarder appearance at the epical region. The epithelial layer contained numerous secreting goblet cells. There were several lymphocytes occupying in between epithelial cells. Below the epithelial layer there was a loose connective tissue area, in which numerous lymphocytes were found scattered. Among the lymphocytes, occasionally cells having eosinophilic granules were also found. Further deep, the submucosal tissue was formed of connective tissue containing thick accumulation of lymphocytes and several eosinophilic granular cells. (Plates 20a – 20b).

4.6 Inflammation

The grouper, *Epinephelus tauvina* exhibited inflammatory response against Freund's complete adjuvant. The response observed was acute inflammatory response. Numerous neutrophils, lymphocytes and also macrophages were seen in the blood capillaries and noticed migrating towards site of injection. A biphasic leucocyte migration pattern, neutrophils and lymphocytes followed by macrophages was evident. Many of them infiltrated to the site of injury. The exudates contained fibrin, neutrophils, lymphocytes and monocytes and those were accumulated at the site of injection. Neither basophils nor eosinophils were noticed at the site of injury. (plates 21a – 21e).

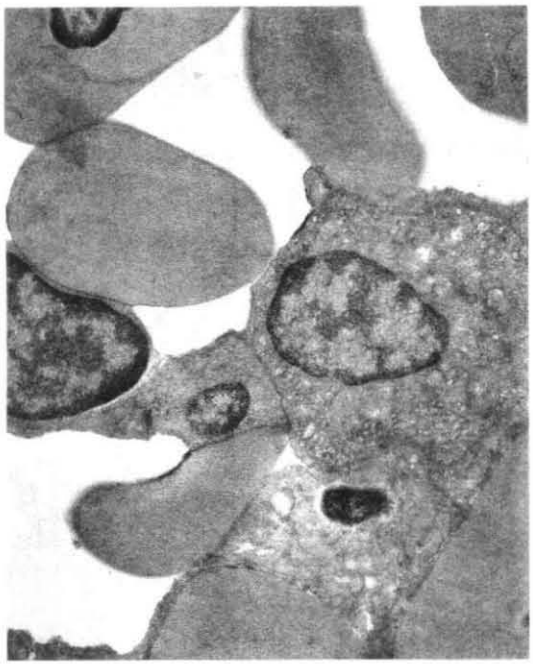


Plate 19a. Electron micrograph of spleen of *E. tauvina* showing spleenic haemopoietic tissue with developing stages of blood cells. (3500 X).

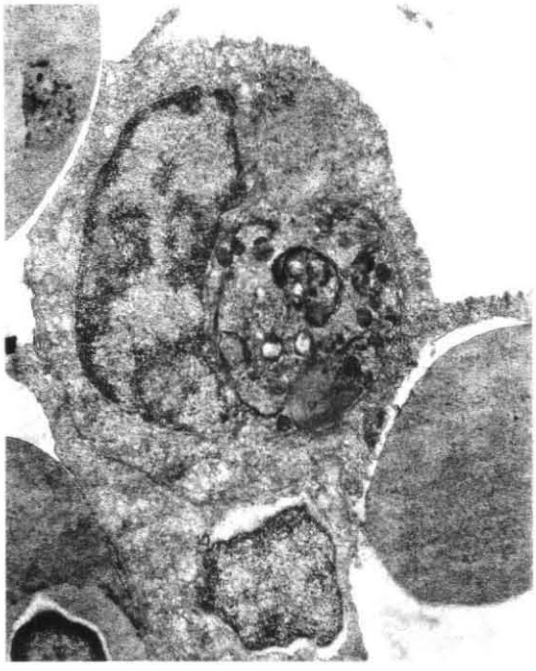


Plate 19b. Electron micrograph of spleen of *E. tauvina* shwoing haemopoietic tissue with developing stages of blood cells.(3500 x).

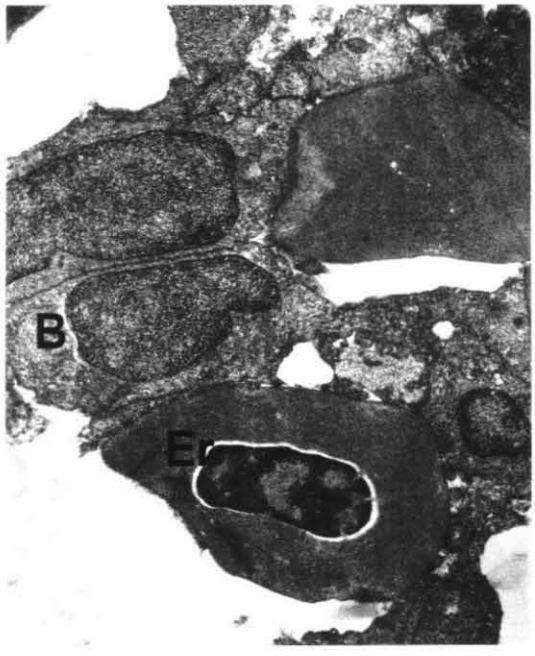


Plate 19c. Electron micrograph of spleen of *E. tauvina* showing haemopoietic tissue with erythrocytes(Er) and blast cells (B). (3500 x).

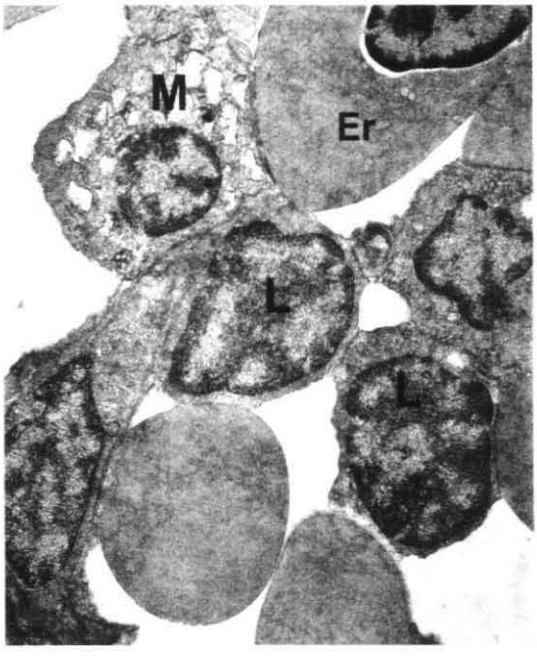


Plate 19d. Electron micrograph of spleen of *E. tauvina* shwoing haemopoietic tissue with lymphoid cells (L), monocytes (M) and erythrocytes (Er). (3500 x).

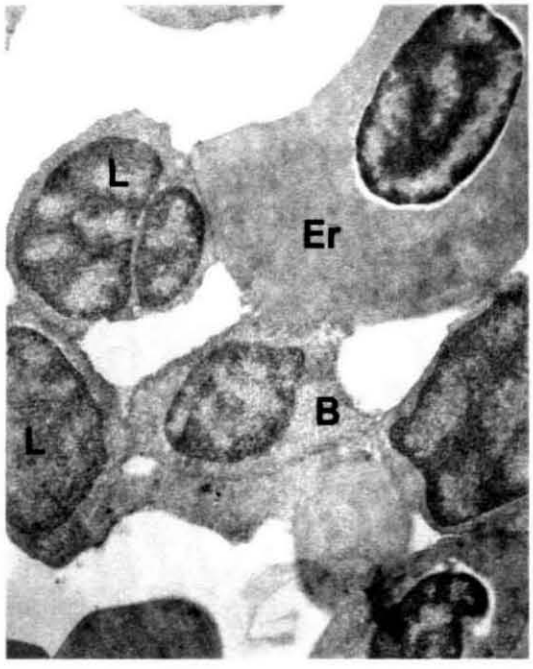


Plate 19e. Electron micrograph of spleen of *E. tauvina* showing spleenic haemopoietic tissue with lymphoblasts(L),erythroblasts (Er) and blast cells (B).(3500 X).

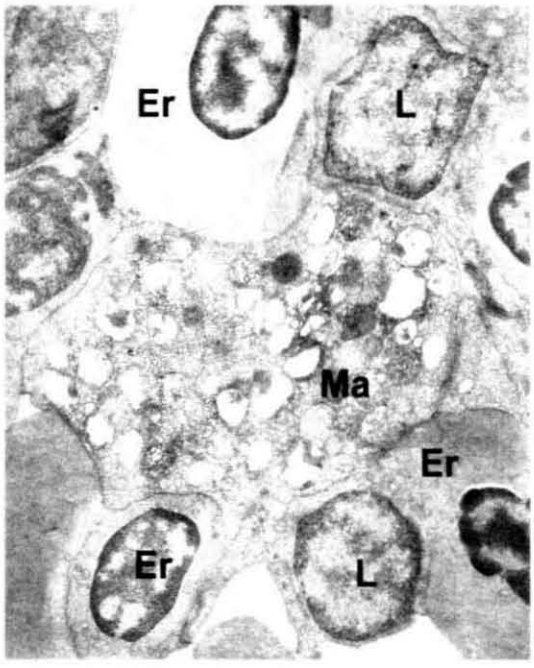


Plate 19f. Electron micrograph of spleen of *E. tauvina* showing haemopoietic tissue with lymphocytes(L), erythroblasts (Er) and macrophages(Ma). (3500 X).

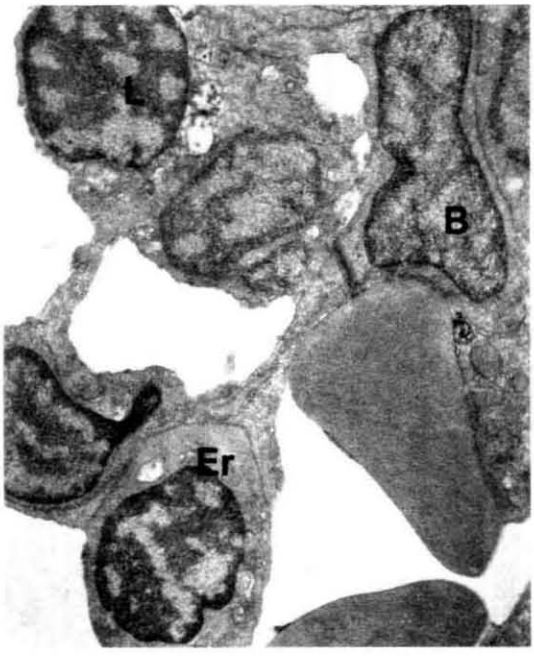


Plate 19g. Electron micrograph of spleen of *E. tauvina* showing haemopoietic tissue depicting erythroblasts (Er), early blast cells (B) and lymphoblasts (L). (3500 X).

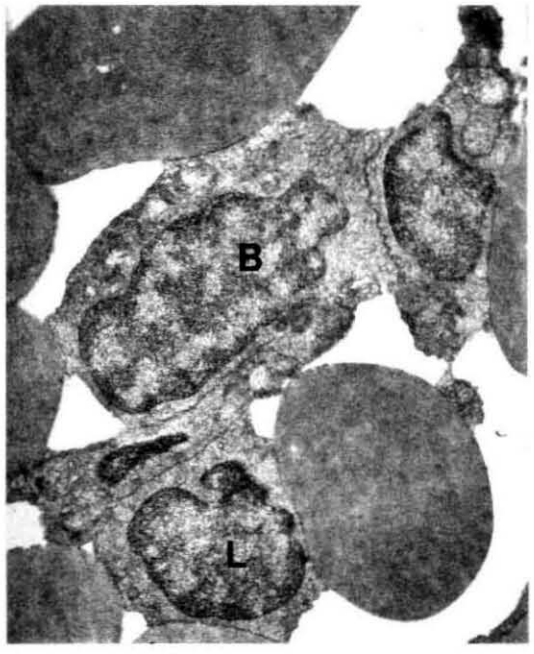


Plate 19h. Electron micrograph of spleen of *E. tauvina* showing spleenic haemopoietic tissue with blast cells (B) and lymphoid cells (L). (5000 X).

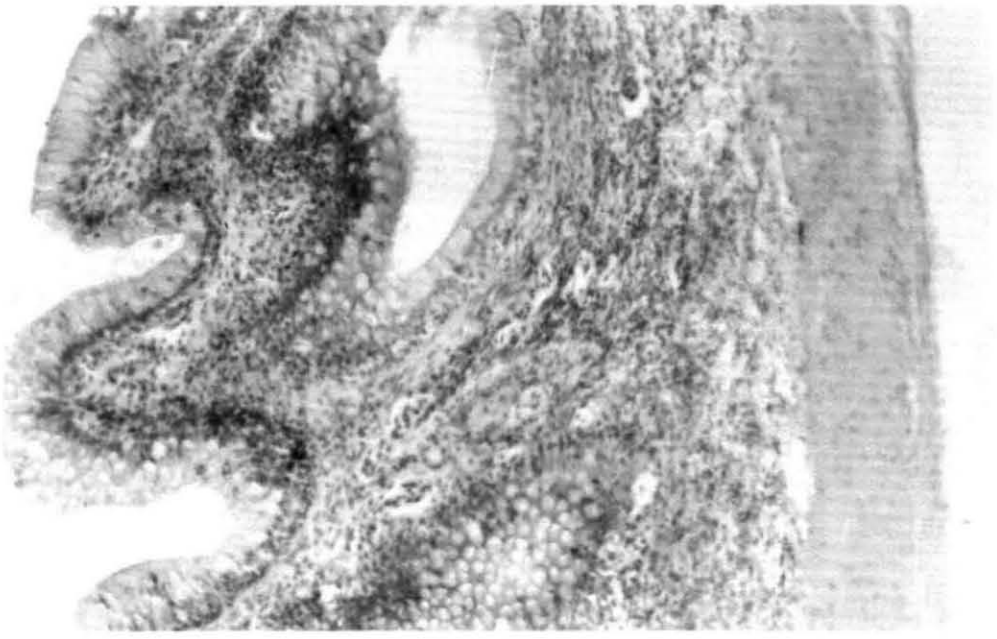


Plate 20a. Section of intestine of *E. tauvina*. The outer surface is lined with columnar epithelum and mucosa is thrown into several folds forming villi. The lamina contains numerous round nucleated cells, which are the lymphocytes. Further deep, more lymhocytes and pink staining eosinophilic granular cells can be seen. H & E, 200 x.

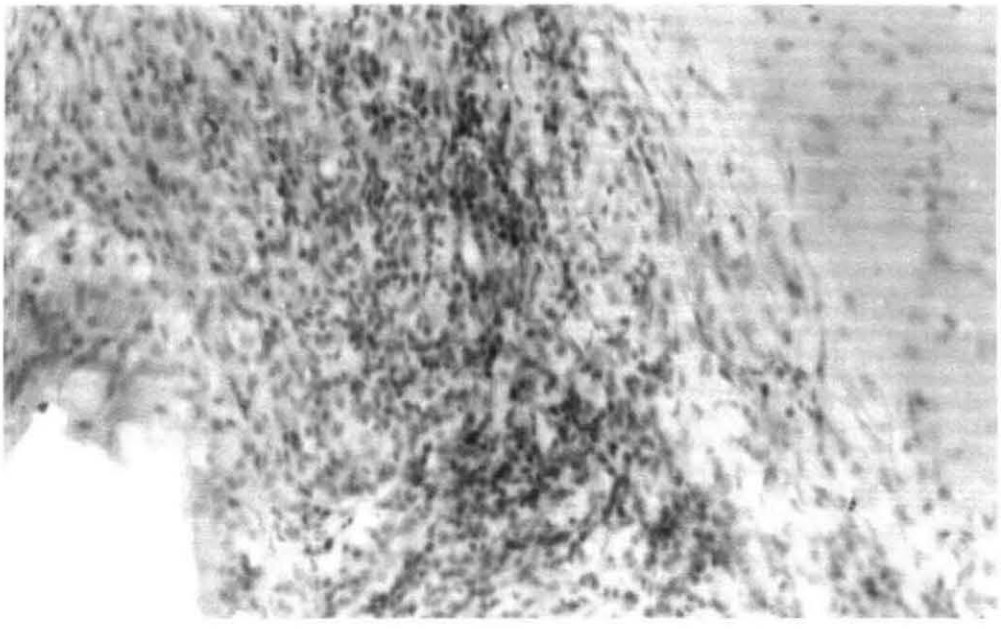


Plate 20b. Section of intestine of *E. tauvina*. Note the mucosal distribution of lymphocytes. H & E, 400 x.

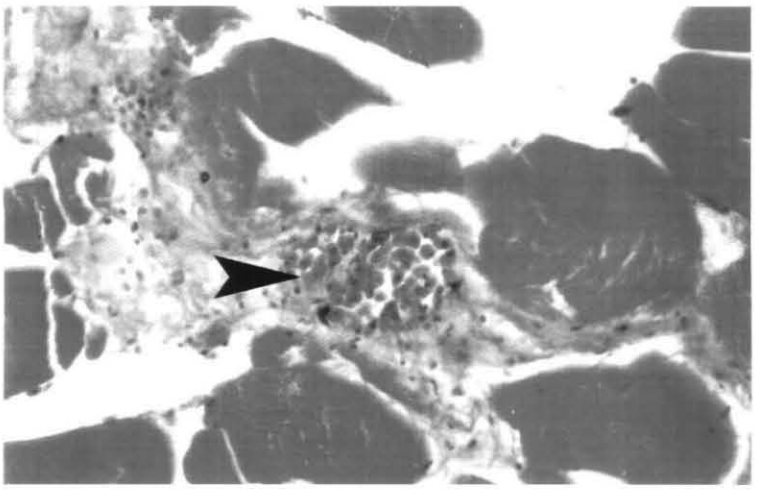


Plate 21a. Section of trunk musculature of *E. tauvina* after 24 h injection with FCA . Note the dilatation of blood vessel (arrowhead) engorged with erythrocytes and leucoctyes. H & E, 400 x.

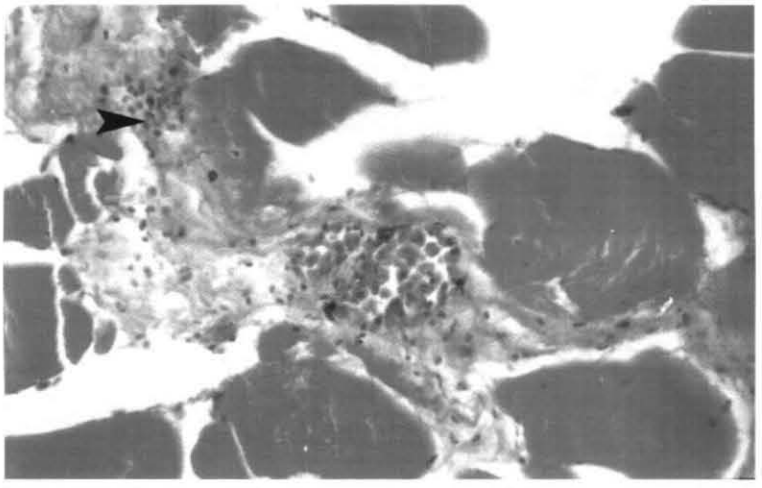


Plate 21b. Section of trunk musculature of *E. tauvina* after 24 h injection with FCA . Note the leucocytes and presence of fibrin (arrowhead). H & E, 400 x.

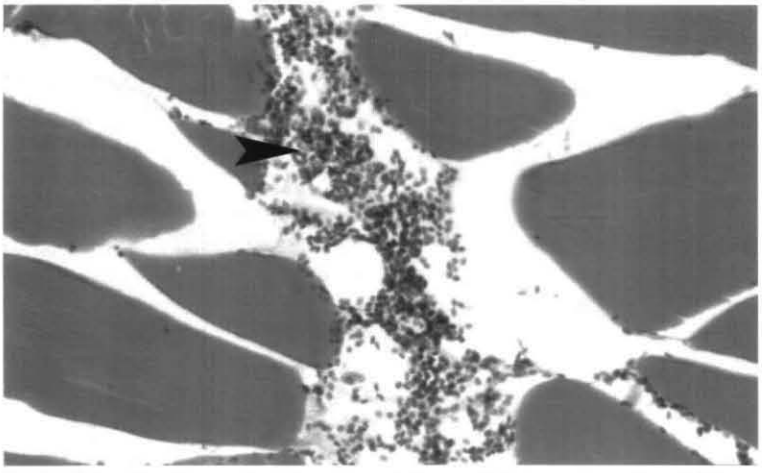


Plate 21c. Section of trunk musculature of *E. tauvina* after 24 h injection with FCA . Note the infiltration of leucocytes in the intermuscular area (arrowhead). H & E, 400 x.

Plate 21d. Section of trunk musculature of *E. tauvina* after 24 h injection with FCA . The accumulation of leucocytes and fibrin can be seen in interstitial area. H & E, 400 x.

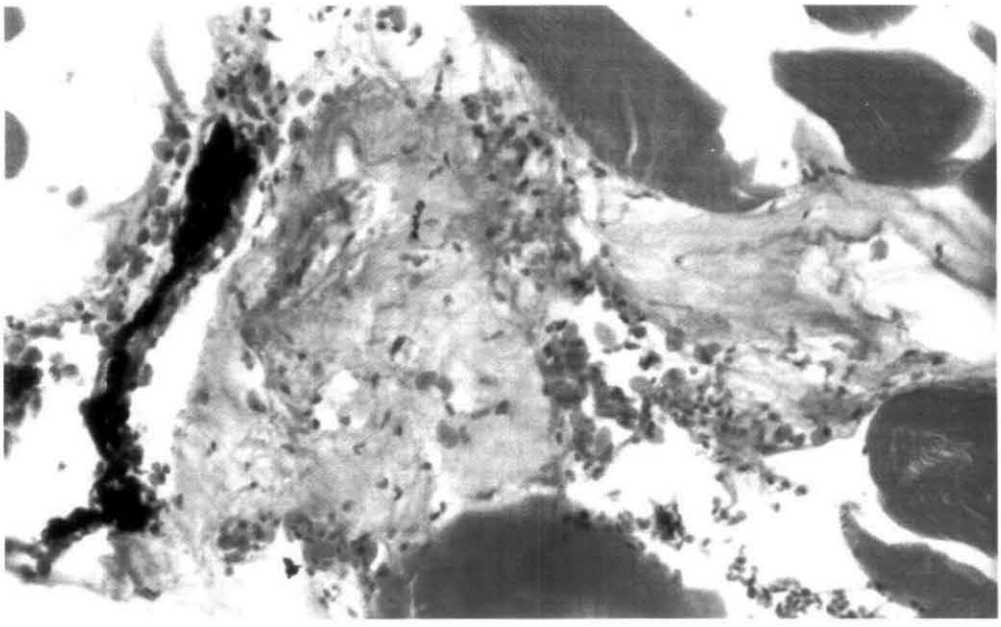


Plate 21e. Section of trunk musculature of *E. tauvina* after 24 h injection with FCA . Note the leucoctyes and fibrin. H & E, 400 x.

4.7 Phagocytosis

Intraperitoneal injection of Indian ink after 24 h showed presence of carbon filled phagocytes in the peritoneum. The phagocytes were moderate in number and approximately 15 to 20 cells were noticed in a high power microscopic field. (Plates 22a – 22c).

The leucocytes collected from peripheral blood showed a phagocytic index, which varied between 29 and 32.5 % and the mean percentage was 31.15 ± 0.46. It was found that majority of the cells were not phagocytic. Non-phagocytic cells were between 67.5 to 71% and the mean value was 68.84 ± 0.47 %. (Table 5 and figure 9).

4.8 PHA-Sensitivity

To evaluate the T cell response PHA was injected intradermally and skin thickness was monitored. There was significant increase in thickness of skin developed after 24 h in caudal peduncle. This was maintained till 72 h. The rate of increase of skin thicknesses were shown significance difference ($p < 0.01$). The skin thickness before and after injection is given in the table 6 and figure 10.

4.9 Rosette Formation

The leucocytes were suspended along with rabbit blood erythrocytes for 24 h at 10^0 C in HBSS and the number of cells forming rosette with erythrocytes were estimated. It was found 39.67 ± 0.71 % of peripheral blood cells formed rosette with rabbit erythrocytes. The rosette formation of leucocytes with rabbit erythrocytes on *Epinephelus tauvina* is given in table 7 and figure 11.

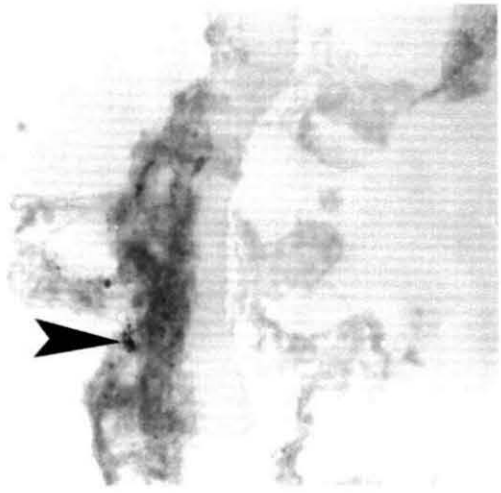


Plate 22a. Portion of intestinal serosa depicting phagocytic cells with carbon particles (arrowhead) after intraperitoneal injection of Indian ink into peritoneal cavity of *E. tauvina*. H & E, 200 x.

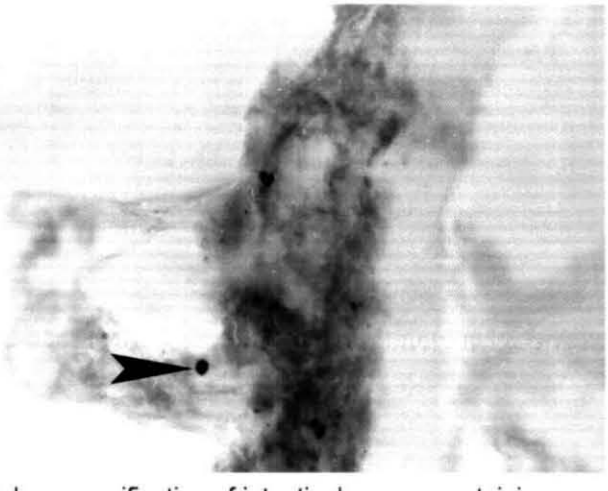


Plate 22b. Higher magnification of intestinal serosa containing phagocytic cells. Note the phagocytes filled with carbon particle appear as dark spot (arrowhead) after intraperitoneal injection of Indian ink into peritoneal cavity of *E. tauvina*. H & E, 400 x.

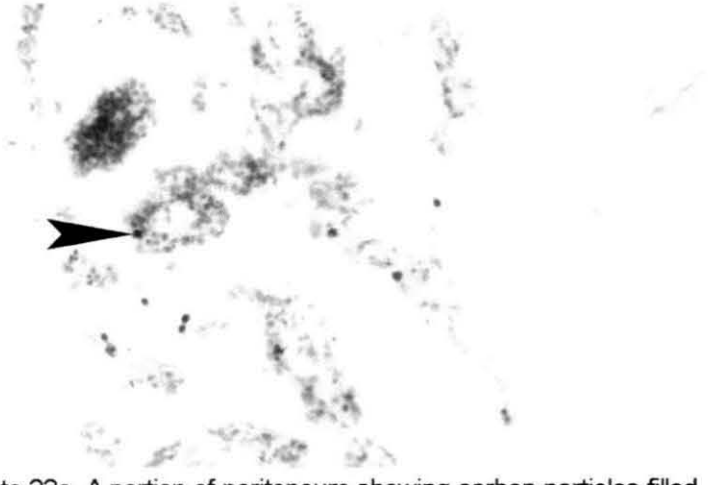


Plate 22c. A portion of peritoneum showing carbon particles filled within phagocytes (arrowhead) after intraperitoneal injection of Indian ink into peritoneal cavity of *E. tauvina*. H & E, 400 x.

Table 5. Phagocytic index in *E. tauvina*.

PHAGOCYTIC INDEX		
No. of leucocytes, which have ingested yeast cells and those without yeast cells		
Total no. of cells counted	Leucocyte with yeast cells (in %)	Leucocyte without yeast cells (in %)
163	32.5	67.5
200	29.0	71.0
190	30.52	69.48
205	31.7	68.3
200	32.5	67.5
205	30.73	69.27
200	31.15	68.85

Mean percentage of phagocytosis	
Type of cells	Mean ± standard error
With yeast cells	31.15 ± 0.46
Without yeast cells	68.84 ± 0.47

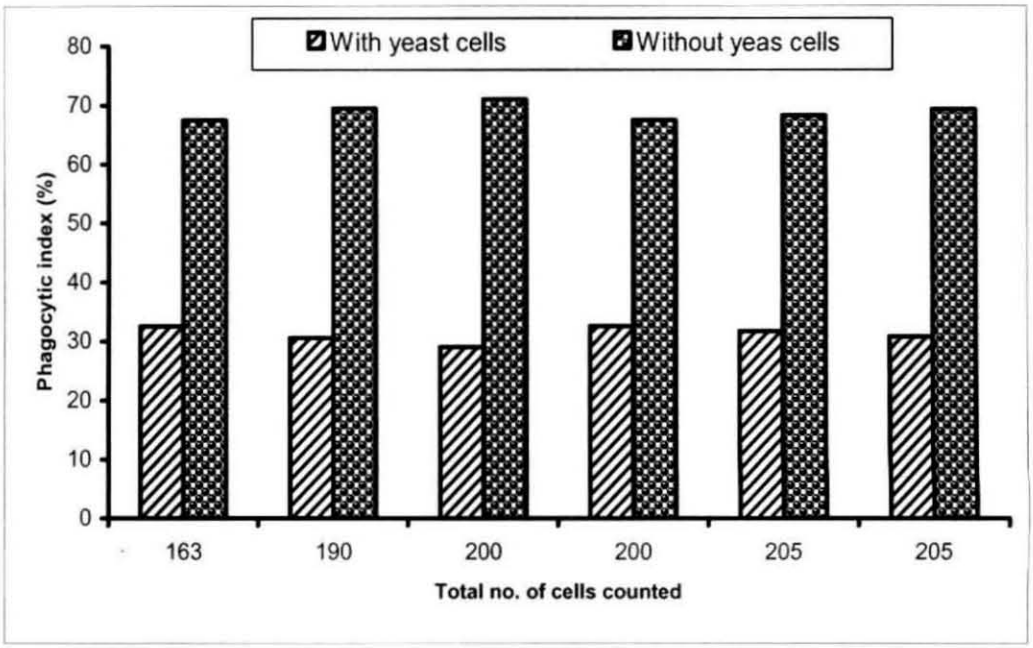


Figure 9. Phagocytic index in *E. tauvina*.

Table 6. Phytohaemaglutinin- M sensitivity response in *E. tauvina*.

PHA- SENSITIVITY					
Skin thickness (in mm)					
Weight of the fish (gm)	Time after injection (hrs)				
	0 hr	24 hrs	48hrs	72 hrs	96 hrs
85	8.93	9.71	9.51	9.42	8.94
100	8.56	9.21	9.05	9.00	8.86
105	8.84	9.32	9.12	9.05	8.95
115	8.77	9.65	9.42	9.32	8.82
130	8.95	9.62	9.41	9.37	8.62
150	8.92	9.71	9.48	9.36	8.95

Mean increase in skin thickness (mm)	
Time after injection (hrs)	Mean ± standard error
0 hr	8.82 ± 0.06
24hrs	9.53 ± 0.1
48hrs	9.32 ± 0.08
72hrs	9.25 ± 0.07
96 hrs	8.86 ± 0.06

The mean increase in skin thickness of various time intervals after injection was compared by one-way ANOVA and resulted significant difference in skin thickness at 1% level ($p < .01$).

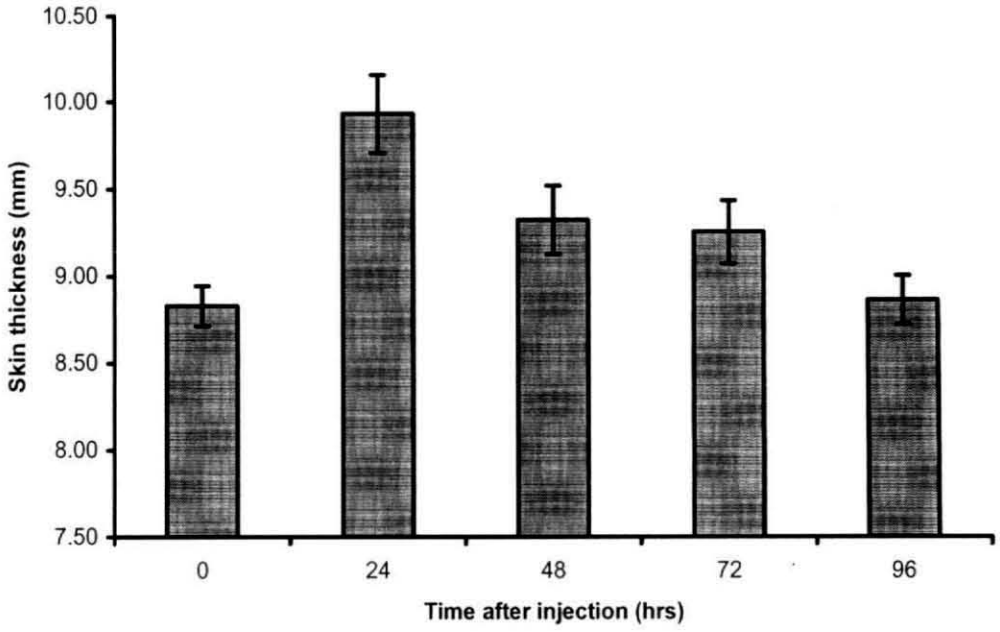


Figure 10. Caudal peduncle thickness in *E. tauvina* before and after injection of phytohaemaglutinin-M.

Table 7. Rosette formation of leucocytes with rabbit erythrocytes on *E. tauvina*.

ROSETTE FORMATION	
Counts of 100 leucocytes for Rosette formation with rabbit erythrocyte	
Cells with rosette (out of 100 cells)	Cells without rosette (out of 100 cells)
39	61
42	58
37	63
40	60
41	59
39	61
Mean count of Rosette forming cells	
Type of cells	Mean ± standard error
Cells with rosette	39.67 ± 0.71
Cells without rosette	60.34 ± 0.71

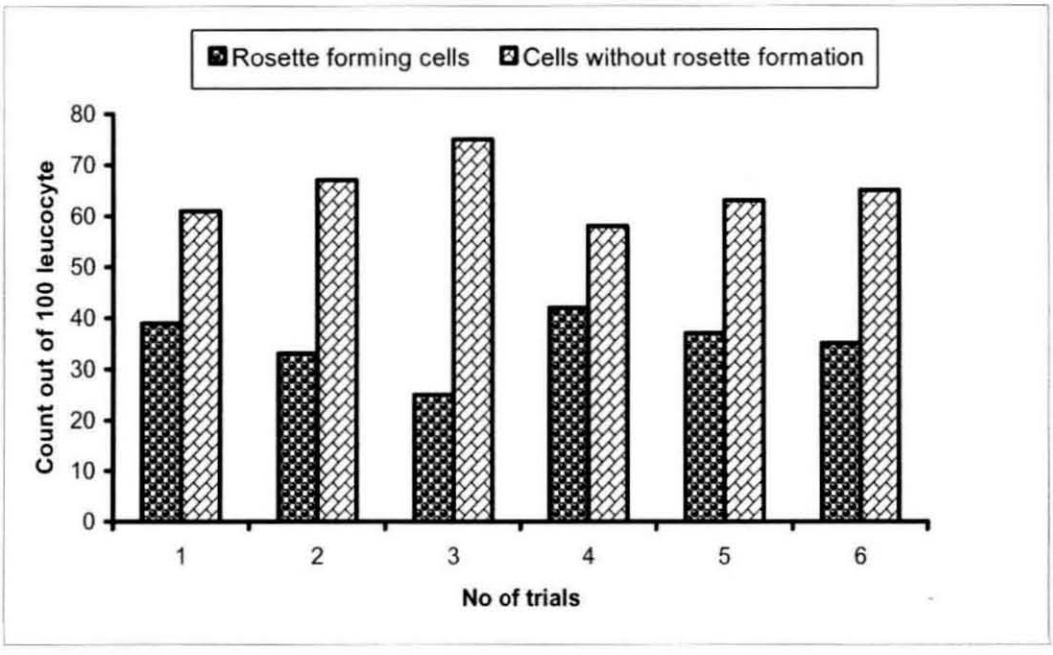


Figure 11. Rosette formation of the leucocytes with rabbit erythrocytes in *E. tauvina*.

DISCUSSION

5. DISCUSSION

5.1 HAEMATOLOGY

In the present work, the Greasy grouper, *Epinephelus tauvina* ranging from 30 to 420 gm body weight were used. Important blood parameters like erythrocyte count (million/mm^3), leucocyte count (1000/mm^3), serum total protein, albumin, globulin and albumin-globulin ratio etc. were estimated. There is a dearth of information regarding the various haematological parameters during the normal as well as pathological conditions of this species. Hence it is essential to build up basic information on the various haematological parameters under normal as well as altered physiological conditions. Similar studies were conducted earlier in various other fish species (Barnhart, 1969; McCarthy *et al.*, 1973; McCarthy *et al.*, 1975). Some of the blood parameters of the rainbow trout, *Salmo gairdneri* (kamloop and shasta varieties) were studied. Erythrocyte count (million/mm^3) and leucocyte count (1000/mm^3) along with many other parameters were estimated from male, female and immature fish of both the varieties. The parameters of immature trout of both varieties were similar. Further, the parameters of male and female trout were also similar with only the erythrocyte count exhibiting a significant difference. However large differences were noticed between the parameters of mature and immature shasta variety. (McCarthy *et al.*, 1973; McCarthy *et al.*, 1975)

In the present study, the total erythrocyte count of the fishes varied from 0.80×10^6 /mm^3 to 13.5×10^6 /mm^3 with mean count of 4.42×10^6/mm^3 ± 0.69. The estimated count was higher than the previous studies conducted on trouts (McCarthy et al., 1973; McCarthy et al., 1975). Total erythrocyte count increased with increase in body weight with minor fluctuations. Total leucocyte count showed the same trend too. Total leucocyte count varied from 32.5 $\times 10^3$/ mm^3 to 150.5 $\times 10^3$/ mm^3 with a mean of 72.75 $\times$ 10^3 / mm^3 ± 0.85. The estimated count was higher than the previous studies carried out in carps and pearlspot (George, 1998; Anikuttan, 2004). George (1998) and Anikuttan (2004) reported a mean leucocyte count of 24.3 $\times$ 10^3 / mm^3 and 36.1 $\times$ 10^3 / mm^3 for the tropical fishes *Labeo rohita* (rohu) and *Etroplus suratensis* (pearl spot) respectively.

Such variation in counts may occur when fishes are studied over a prolonged period of time and the fish being exposed to varying conditions of salinity, temperature and other physiological factors (Blaxhall & Daisley, 1973; Ellsaesser & Clem, 1985; Ellis, 1976). However, first hand information has been obtained on the haematological parameters of *Epinephelus tauvina*.

5.2 SERUM FACTORS

The total serum protein values showed wide variation, between 0.40 and 3.329 g % with a mean of 1.99 ± 0.04 g %. The usual serum protein values encountered for fishes are 0.17 to 0.61 g % (Roberts and Ellis, 2003). Serum protein levels are often subject to fluctuations (Ellis, 2003). Most of these aspects have been studied in the fishes of temperate region (McCarthy *et al.*, 1975; Ellsaesser and Clem, 1986, Roberts and Ellis, 2003). Studies on similar aspects have been initiated on a tropical marine fish *Epinephelus tauvina* here.

The present study revealed 8 – 10 electrophoretic separation bands in the serum of the grouper. This is in conformity with the earlier studies (Schaperclaus, 1991). In *E. tauvina,* the first moving fraction (albumin) contributed more than 22 % and the last fraction, which probably contained immunoglobulin, more than 18%. Ellis (2003) reported that the albumin fraction contributed 6 to 15 % of the total serum protein. Since in the present study, complete fractionization of serum was not achieved, it was difficult to estimate the exact percentage of immunoglobulin. However the fractions containing immunoglobulin formed approximately 18%.

5.3 DIFFERENTIAL COUNT

Several investigations on the nature of fish leucocytes have been carried out. Unfortunately, such investigations have not produced uniform information and have resulted in numerous nomenclature and classification systems of fish leucocytes (Drzewina, 1911; Jordan, 1926; Ellis, 1975; Ainsworth, 1992; Zapata *et al.*, 1996; Ellis, 2003; Moore and Hawke, 2004). In the present work, leucocytes are broadly classified into granulocytes, monocytes, lymphocytes and thrombocytes.

The cells having granules in cytoplasm were classified as granulocytes. Two types of granulocytes were observed: one with mild basophilic granules (neutrophil) and the other have mild eosinophilic granules. Both the types have more or less same structure as described by Ellis (1975, 2003). Cells of such structure were classified as type I leucocytes or neutrophils and their number varied between 6 -8 % (Ellis, 2003). Different species of teleosts have varying number of granulocytes. In goldfish it was reported that neutrophils varied from 5-12% to 1-2% (Watson *et al.,* 1963; Weinreb & Weinreb, 1967). In brown trout the neutrophil percentage formed 0 -25% of the total leucocytes (Blaxhall & Daisley, 1973). In the present work, neutrophil numbers varied between 19 - 32 % and the mean percentage was 26.67± 2.06 of the total peripheral blood leucocytes. The count of neutrophils in the present study was much higher than the earlier reports. Similar results have been reported by Blaxhall & Daisley (1973) in the brown trout. Presence of eosinophils in teleosts is controversial (Ellis, 2003). However, in the present observations, there were cells with eosinophilic granules and their percentage varied from 3 to 7% with a mean of 5.16 ± 0.65. Reports about the presence of eosinophils in fishes like *Carassius auratus* (8%), *Fundulus heteroclitus* (3%), *F. majalis (*5.3%) and *Cyprinodon variegates* (2.3%) do exist (Loewenthal, 1928; Gardner & Yevich, 1969). However present finding is lower than the finding made by Saunders (1968) in which eosinophils of Labridae species formed 28 - 41% of the total leucocyte count.

The monocyte count varied between 3 to 11% and the mean count was 6.33 ± 1.30 of the total blood leucocytes. The count was higher than 0.1% as reported by Ellis *et al.* (1976) in plaice blood leucocyte. Few reports are available on the differential counts of monocyte which makes it difficult for an accepted comparison.

The lymphocyte count varied between 54 to 70% and the mean count was 61.83 ± 2.67 of the total peripheral blood leucocytes. The lymphocyte count estimated in, *E. tauvina* was higher than the reported 30% count for goldfish by Watson *et al.* (1963) and is lower than the reported 71-82% count in the goldfish as estimated by Weinreb & Weinreb (1969). For blood smear preparation heparin solution was used to stabilize thrombocytes. Similar technique was used by Ellis & Parkhouse (1975) and

Ellis (1976). The ratio of thrombocyte: lymphocyte was 1:3 in plaice blood (Ellis & Parkhouse, 1975; Ellis, 1976). Many authors are of the view that the spent thrombocytes may be confused with lymphocyte which may result in high lymphocyte values (Watson *et al.*, 1963; Weinreb & Weinreb, 1969; Blaxhall and Daisley, 1973; McCarthy *et al.*, 1973; Ellis & Parkhouse, 1975; Ellis, 1977).

5.4 LEUCOCYTES

5.4.1 Granulocytes

Several studies have indicated the presence of three types of granulocytes in teleosts: heterophils, acidophils and basophils (Ellis, 1977; Rowley *et al.*, 1988; Hine, 1992; Ainsworth, 1992; Zapata *et al.*, 1996). However, this has not been noticed in all teleosts. Some studies even highlighted very exceptional findings. In plaice, *Pleuronectes platessa* (Ellis, 1976) and the eel, *Anguilla japonica* (Kusuda and Ikeda, 1987), only granulocyte heterophils have been reported. Fishes like channel catfish, *Ictalurus punctatus* (Cannon *et al.*, 1980; Ainsworth & Dexiang, 1990), South African catfish, *Clarias gariepinus* (Savage, 1983), roach, *Rutilus rutilus* (Hoole and Arme,1982), porcupine fish, *Diodon hystrix* (Radhakrishnan *et al.*, 1976) too have only heterophils. Even in the primitive fish, modern river lamprey, *Lampetra fluviatilis* (Page and Rowley, 1983) only heterophils are present. In the present study, mainly two types of granulocytes were noticed. These are neutrophils and eosinophils. The present finding is in conformity with earlier works on the eels (*Anguilla australis, A. dieffenbachii* and *A. anguilla*) (Hine *et al.*, 1986a,b), nurse shark, *Ginglymostoma cirratum* (Hyder *et al.*, 1983), stripped bass, *Morone saxatilis* (Bodammer, 1986) and also on tilapia, *Oreochromis mossambicus* (Doggett *et al.*, 1987). Eosinophil has been reported as the only granulocyte in elasmobranchs (Mainwaring and Rowley, 1985). Such findings have not been noticed in teleosts including *E. tauvina* in the present study.

In the present study on *E. tauvina,* neutrophils had eccentric, round or slightly oval shaped nuclei. Some neutrophils even had bilobed nucleus. The cytoplasm appeared white to whitish gray. This finding is in agreement with numerous studies conducted earlier in several teleosts where it was noticed that neutrophils have white to

whitish-gray cytoplasm and an eccentric round to slightly oval bilobed nucleus when stained with Romanowsky dyes (Ellis, 1976; Cannon *et al.*, 1980; Breazile *et al.*, 1982; Ellsaesser *et al.*, 1985; Kusuda and Ikeda, 1987; Ellis and Youson, 1989; Ainsworth and Dexiang, 1990; Ainsworth, 1992). In the present work cells with round/oval shaped nuclei were commonly observed. Some of the neutrophils had lobed and oval nuclei. This is agreeable with the earlier investigation on L2 granulocyte of roach, *Rutilus rutilus* (Hoole and Arme, 1982) and with other teleosts (Ellis, 1976; Cannon *et al.*, 1980; Breazile *et al.*, 1982; Ellsaesser *et al.*, 1985; Kusuda and Ikeda, 1987; Ellis and Youson, 1989; Ainsworth & Dexiang, 1990; Ainsworth, 1992). In *Chimaera monstrosa* the heterophils are irregular in shape with a horse-shoe like nucleus (Mattisson and Fange, 1986). In the present study on *E. tauvina,* polymorphonuclear neutrophils were also noticed similar to that of trouts and dogfish, *Scyliorhinus canicula* (Finn and Nielson, 1971a,b; Parish *et al.*, 1986).

Granules are considered the most common differentiating feature of the cytoplasm of teleost neutrophils (Ellis,1975, 2003; Ferguson, 1975; Cannon *et al.,*1980; Fange and Mattisson, 1981; Ellsaesser *et al.*, 1984, 1985; Suzuki, 1984; Hine *et al.*, 1986; Temkin and McMillan, 1986; Burrows and Fletcher, 1987; Hine and Wain, 1987; Rowley *et al.*, 1988; Doggett & Harris, 1989; Ellis and Youson, 1989; Fujimaki and Isoda, 1990; Ainsworth, 1992; Zapata *et al.*, 1996; Moore and Hawke, 2004). In the present study also, granules serve as one of the most significant features in the cytoplasm of neutrophils and is comparable with the earlier works on fish neutrophils reported above. Many workers studied the chemical nature of the granules present in neutrophil cytoplasm. The objectives of such studies were to understand the functional activities of granules if any. In the study on *E. tauvina,* important histochemical activities were tested. These were acid phosphatase, peroxidase, periodic acid Schiff reaction (PAS) and Sudan black staining. The cytochemical studies showed many of the neutrophils as positive for PAS, peroxidase and Sudan black stain. Some were acid phosphatase positive. Many of the earlier cytochemical studies in several fishes gave varying results. One of the reasons for such varying results would have been the improper selection of enzymes for their study. Uniformity was not observed for the enzymes to be tested for the granule characterization. In the present study, the above

four mentioned chemical constituents were selected. The presence of PAS positive neutrophil cytoplasmic granules in the present study was agreeable with earlier studies conducted in plaice (Ellis, 1975, 2003). Similarly, positive peroxidase reaction was observed in the present study was agreeable with the earlier studies in channel catfish, rockfish (*Sebastes schlegeli*), rainbow trout (*Oncorhynchus mykiss*), goldfish and plaice (Ellis, 1975; Cannon *et al.,* 1980; Suzuki, 1984; Temkin and McMillan, 1986; Ellis, 2003), whereas negative peroxidase activity was noticed in eels (Hine *et al.*, 1986). Positive reaction in peroxidase indicates antibacterial and phagocytic properties of the neutrophils. Sudanophilic neutrophils in the present study was agreeable with earlier studies in channel catfish (Ellsaesser *et al.*, 1984, 1985; Rowley *et al.*, 1988; Moore and Hawke, 2004), turbot (*Scophthalmus maximus)* (Burrows and Fletcher, 1987) and also in plaice (Ellis, 1975,2003). Mild, positive acid phosphatase reaction in the present study is agreeable with the findings in eels, *Anguilla anguilla*, *A. australis* and *A. dieffenbachii* (Hine *et al.*, 1986) and also in plaice (Ellis, 1975, 2003).

Detailed structural configuration of the neutrophil cytoplasmic granules could be better understood ultrastructurally. In the present study, the neutrophil contained numerous, uniformly homogenous, prominent, round or oval, electron dense, cytoplasmic granules. The present findings were in complete agreement with the earlier studies in plaice, river lamprey and common carp (Ferguson, 1975, 1976; Page and Rowley, 1983; Cenini, 1984). In carp only oval granules were noticed (Cenini, 1984). Heterogeneity in granules in the cytoplasm of neutrophils was probably related to cell maturity rather than sub population of cells (Ainsworth, 1992).

Besides the above granules, some granules having vacuole in the centre with electron dense periphery were also observed in some granulocytes. Earlier workers reported such granules with fibrous material or crystalloid inclusion in the neutrophils of elasmobranchs and plaice (Ferguson, 1976; Morrow and Pulsford, 1980; Fange and Mattisson, 1981; Hyder *et al.*, 1983; Chiba *et al.*, 1988).

The staining characters and ultrastructure of the granules indicate the possible presence of two types of granulocytes. Since the staining differences among the granules are probably low, it is difficult to differentiate the granulocytes into two

types from the blood smears. The staining characteristics in light microscopy largely depend on the pH of the stain. The staining cannot differentiate granules, which have minor isoelectric pH differences. But the ultrastructural differences point out that these cells belong to two different classes.

The presence of the fibrous or crystalloid inclusion in vacuolated granules seems mysterious. The crystalloid inclusion is considered as a characteristic feature of eosinophils in higher vertebrates and the same is true for fish also (Kelenyi and Nemeth, 1969). The above cited authors pointed out clearly that crystalloids of the eosinophils could be attributed to the peroxidase activity at pH 11-12 rather than at pH 7-8. However, Ferguson (1975) and Fange and Mattisson (1981) argued that such crystalloid inclusions were part of neutrophils. The presence of two types of granules clearly indicated that the granulocytes do not belong to a single class /group in the case of *E. tauvina*.

5. 4. 2 Lymphocytes

In the present study, two types of lymphocytes, one large and the other small were observed. These two types were not from separate populations but represent different states of function. The finding was very similar to earlier works in gold fish, rainbow trout and plaice (Watson *et al.*, 1963; Weinreb and Weinreb, 1969; McCarthy *et al.*, 1973; Ellis and Parkhouse, 1975; Ellis, 1976).

In large lymphocytes, nucleus occupied the major portion of the cell leaving a narrow rim of peripheral basophilic cytoplasm. While small lymphocytes were occupied completely by large nucleus. Weinreb (1963) and Ellis (1976) had observed such lymphocytes in their study on goldfish and plaice.

The fine structure of lymphocyte of *E. tauvina* revealed a very prominent, large nucleus containing both euchromatin and heterochromatin. A few mitochondria, some amount of rough endoplasmic reticulum and numerous polyribosomes were noticed in cytoplasm. Similar structures were also recorded earlier in goldfish and plaice

(Weinreb, 1963; Ferguson, 1976). The above findings were very much in agreement with the findings in mammalian lymphocytes (White, 1963).

5.4.3 Monocytes

In the present study on *E. tauvina,* cells designated as monocytes were having large eccentric nucleus. The cytoplasm appeared foamy or vacuolated with occasional granules. The granules were mildly positive for PAS and acid phosphatase. The findings of the present study were very similar to earlier work in plaice (Ellis *et al.*, 1976).

The fine structure of monocytes in the present study revealed abundant cytoplasm with numerous mitochondria, vacuoles, lysosomes and with few stacks of rough endoplasmic reticulum and some Golgi apparatus. The nucleus was situated eccentrically with chromatin dispersed marginally. The above findings were similar to earlier studies in plaice (Ellis *et al.*, 1976; Ferguson, 1976).

Monocytes of *E. tauvina* may be capable of phagocytosis and they may be able to ingest colloidal particles as reported by earlier workers (Weinreb and Weinreb, 1969; Ellis *et al.*, 1976; Ellis, 2003). There is need for further investigation.

5.4.4 Thrombocytes

The thrombocytes of *E. tauvina* appeared as elongated, spindle shaped cells with nucleus at the centre, surrounded by cytoplasm containing vacuoles. Though Ellis (1976) described four different types of thrombocytes in fish, such differentiation was not noticed in the present study.

The fine structure of thrombocytes revealed cytoplasm containing numerous vacuoles. These vacuoles were interconnected and opened to the exterior through fenestrae. The nucleus was large and contained both euchromatin and heterochromatin. The above findings in *E. tauvina* were similar to the earlier study in plaice thrombocytes (Ferguson, 1976).

5.5 LYMPHOID ORGANS

5.5.1 Thymus

Thymus gland in *E. tauvina* is a well developed, paired organ present on the dorsolateral region of the gill chamber. The finding of the present study was similar to the earlier investigations in numerous teleost species (Grace and Manning, 1980; Zapata, 1981; Chilmonczyk, 1992; Moore and Hawke, 2004).

The entire thymus was surrounded by a connective tissue capsule that sent several trabeculae into thymic parenchyma. The trabecula contained fibroblasts, collagen fibres and myo-epithelial cells. The thymic parenchyma also contained numerous lymphocytes, myoid cells and epithelial cells. Differentiation of thymic parenchyma into cortex and medulla had not been seen. Grossly the gland is not lobulated. The facts observed in the present study were similar to the earlier work in other fishes (Lagabrielle, 1938; Good *et al.*, 1966; Sailendri and Muthukkaruppan, 1975; Ghoneum and Egami, 1982; Teshima and Tomonaga, 1986; Chilmonczyk, 1992).

Special stains were used in the present study to understand the structural details. The connective tissue consisting of abundant fibroblasts and collagen was seen in the trabeculae and capsules. These trabeculae projected into the thymus parenchyma bearing blood vessels and nerves. The gland was also divided into several small lobules. The stromal support of the lobule consisted of mainly the reticular connective tissue and some collagen fibrils. These lobules contained lymphoblasts and interdigitating epithelial cells. Some macrophages, myoid cells and myofibrils were also present in the lobules. The facts observed in this study were in agreement with many authors, who had described the structure of thymus in several fishes earlier (Lagabrielle, 1938; Good *et al.*, 1966; Mulcahy, 1970; Henry, 1972; Sailendri and Muthukkaruppan, 1975; Belova, 1976; Tamura and Honma, 1977; Zapata, 1980; Tamura *et al.*, 1981; Ghoneum and Egami, 1982; Chilmonczyk, 1983; Gorgollon, 1983; Pulsford *et al.*, 1984; Fange and Pulsford, 1985; Teshima and Tomonaga, 1986; Castillo *et al.*, 1990; Chantanachookhin *et al.*, 1991; Chilmonczyk, 1992; Pulsford *et al.*, 1991; Iwama and Nakanishi, 1996).

The fine structure of thymus of *E. tauvina* showed many blood capillaries, surrounded by epithelial cells. In the capillaries, the lumen was filled with blood cells. Most of the blood capillaries are lined by endothelial cells with tight junctions. Occasionally some of the epithelial cells were fenestrated in appearance while others had electron dense cytoplasm. Outside the epithelial cells, numerous lymphoblasts were seen. The above findings were in conformity with earlier studies (Mulcahy, 1970; Zapata, 1980; Botham and Manning, 1981; Tatner and Manning, 1983; Pulsford *et al.*, 1984; Castillo *et al.*, 1991; Chilmonczyk, 1992). Several sub–populations of the epithelial cells were reported based on morphology, fine structure, and histochemical activities by Castillo *et al.* (1990) and Pulsford *et al.* (1991). The present study could not divide the epithelial cells into subpopulations. The species *E. tauvina* may not have such divisions of epithelial cells. This could be better understood when further study will be carried out on this species.

5. 5. 2 Kidney

Kidneys of the grouper *E. tauvina* were capsulated, dark-red organs extending ventrally along the vertebral column. The location and colour pattern of the species in the study was in agreement with earlier findings in other teleosts (Ellis, 1974; Ellis and de Sousa, 1974; Ferguson, 1975; Sailendri and Muthukkaruppan, 1975; Ellis *et al.*, 1976; Ellis, 1977; Zapata, 1979; Agius, 1980; Grace and Manning, 1980; Secombes and Manning, 1980; Botham and Manning, 1981; Bly,1985; Tatner and Manning, 1985; Razquin *et al.*, 1990; Chantanachookhin *et al.*, 1991; Zapata *et al.*, 1996; Press and Evensen, 1999; Ellis, 2003; Roberts and Ellis, 2003; Moore and Hawke, 2004). The kidneys of the grouper *E. tauvina* were paired organs and each separated into the anterior and posterior kidney. The present work was similar to the earlier findings in carp (Botham and Manning, 1981), channel catfish (Moore and Hawke, 2004), and other marine fishes (Chantanachookhin *et al.*, 1991).

The light microscopy of the thin section of the anterior and posterior kidney of *E. tauvina* was carried out to locate common structural and functional details. The anterior kidney of the species *E. tauvina* consisted of exclusively haemopoietic tissues while the posterior kidney was filled with haemopoietic tissues in the interstitial area that

surround (support) the nephrons. The findings of the study were similar to that in tilapia, plaice, channel catfish and other marine teleosts (Ellis and de Sousa, 1974; Sailendri and Muthukkaruppan, 1975; Chantanachookhin *et al.*, 1991; Petri-Hanson and Ainsworth, 2001). The anterior kidney in *E. tauvina* is mainly for haemopoietic activities whereas the posterior kidney showed renal function as in the case of other species. The presence of numerous blast cells in the stromal tissue of posterior kidney was reported by Chantanachookhin *et al.* (1991). This is indicative of the occurrence of haemopoiesis in the posterior kidney along with other renal activities. Numerous blood sinuses lined with endothelial cells were observed in both anterior as well as posterior kidney in *E. tauvina*. The endothelial lining in the sinuses acts as filter in addition to the foci of haemopoietic activity. The observations presented here agreed well with the observations of several earlier workers cited above. The developing leucocytes were also observed within sinuses and outside sinuses. Cells were scattered randomly throughout the stroma. The findings of the present work was in conformity with the previous works (Ellis and de Sousa, 1974; Sailendri and Muthukkaruppan, 1975; Ellis *et al.*, 1976; Zapata, 1979; Agius, 1980; Grace and Manning, 1980; Secombes and Manning, 1980; Botham and Manning, 1981; Bly, 1985; Tatner and Manning, 1985; Chantanachookhin *et al.*, 1991; Zapata *et al.*, 1996; Press and Evensen, 1999; Ellis, 2003; Roberts and Ellis, 2003; Moore and Hawke, 2004). Another cellular structure observed in the greasy grouper was the melanomacrophage centers throughout the haemopoietic tissue. Similar structures were also reported in earlier studies in all teleosts (Ellis and de Sousa, 1974; Sailendri and Muthukkaruppan, 1975; Ellis, 1977; Agius, 1980; Grace and Manning, 1980; Secombes and Manning, 1980; Botham and Manning, 1981; Bly, 1985; Tatner and Manning, 1985; Razquin *et al.*, 1990; Chantanachookhin *et al.*, 1991) and was later accepted as one of the structural characteristics of teleosts (Roberts, 1975b; Zapata *et al.*, 1996; Press and Evensen, 1999; Ellis, 2003; Roberts and Ellis, 2003; Moore and Hawke, 2004). Melanomacrophage centres vary in their degree of organization, depending on species. In lower teleosts, e.g. trout there are clusters of dark cells distributed throughout the haemopoietic tissue and the degree of melanization varies with age but the pigment remains dark brown or black and bears melanin (Roberts, 1975b). In the greasy grouper

E. tauvina, there is the presence of nodular, pale coloured melanomacrophage centres. Such centers were located in association with vascular channels and have lymphocytes inside. The findings of the present study agreed with the structure of melanomacrophage centres found in higher teleosts like carp and plaice (Ellis and de Sousa, 1974; Roberts, 1975b; Zapata *et al.*, 1996; Press and Evensen, 1999; Ellis, 2003; Roberts and Ellis, 2003; Moore and Hawke, 2004). Edelstein (1971) suggested that these pigments were formed of melanin or age pigments. Histochemically this pale colouration in higher teleosts was due to lipofuscin. The pale colouration noticed in the greasy grouper *E. tauvina* melanomacrophage centers need to be examined further.

The fine structure of kidney of *E. tauvina* observed in different magnifications revealed the following. Numerous lymphoblasts and granulocytes were seen in the cellular matrix. This finding was in agreement with the earlier studies in viviparous teleost, *Zoarces viviparous*, carp and in other teleosts (Bly, 1985; Imagawa *et al.*, 1991; Press and Evensen, 1999). Some of the granulocytes observed in the present study contained crystalloid granules while others had dense cytoplasmic granules. The mesenchymal tissues along with fibroblasts were evident. The blood sinuses could be clearly seen. The structural details noticed in the present study may probably indicate more specialization and organization of haemopoietic tissue similar to the observations of Press and Evensen (1999).

5. 5. 3 Spleen

In the present study, the spleen of *E. tauvina* is a dark red, elongated, cylindrical rod like structure situated very near to the greater curvature of the stomach or flexure of intestine. The findings were very similar to the earlier study in teleosts (Bond, 1979). Such well developed spleen was not reported in primitive hagfish and lampreys but many reports confirmed the presence of spleen like organ based on histological studies. In lampreys, lymphohaemopoietic accumulations along the entire gut are involved in antigen trapping and processing as in higher vertebrates (Good *et al.*, 1966). Otherwise, a clear isolated organ was reported to be present in elasmobranchs very close to gut or pancreas. The location of spleen in different group of fish was very close to either stomach or intestine (Bond, 1979; Moore and Hawke, 2004).

The typical, elongated, cylindrical, rod like structure of *E. tauvina* spleen was an exceptional observation in the present study and differs from earlier findings (Bond, 1979). Bond (1979) reported the teleost spleen in general as triangular in shape. The spleen of *E. tauvina* was a single organ with no lobes and this observation was in agreement with earlier reports (Robert and Ellis, 2003; Iwama and Nakanishi, 1996). The dark red colour of *E. tauvina* spleen observed in the study was very similar to the colour pattern of several species (Bond, 1979; Moore and Hawke, 2004).

The light microscopy of *E. tauvina* spleen revealed important structural details. The emerging potential of *E. tauvina* for tropical mariculture and its wide intensification may pose serious health concerns and hence there is a need to document the normal structural details in advance similar to other marine species (Chantanachookhin *et al.*, 1991). It is believed that the present documentation is probably the first such effort as far as tropical marine species is concerned. The spleens of *E. tauvina* is composed of both white and red pulp but are not well differentiated and were found scattered throughout the spleen. The above observation is in agreement with Zapata (1982) and Kennedy-Stoskopf (1993). The random presence of white and red pulp in *E. tauvina* as indicated in the present work may probably enable the spleen to house more blood cells rather than in spleens of elasmobranchs (Tomonaga *et al.*, 1984; Fange and Nilsson, 1985; Zapata and Cooper, 1990 where clear differentiations of red and white pulp occurred) and lungfishes (Good *et al.*, 1966; Fange, 1982). The spleen was surrounded by spleenic capsule formed of fibrous connective tissue. The present observation was in conformity with that of plaice (Ferguson, 1976). The spleenic capsule composed of collagen is not a common structural phenomenon in primitive fishes and modern elasmobranches (Anderson, 1974; Page and Rowley, 1982; Zapata, 1982; Fange, 1984; Bly, 1985; Felten *et al.*, 1985; Payan and Goetzl, 1985; Navarrao, 1987; Ardavin and Zapata, 1988; Quesada *et al.*, 1990; Zapata and Cooper, 1990; Kennedy-Stoskopf, 1993; Lloyd-Evans, 1993; Press *et al.*, 1994). Random positioning of mesenchyma and parenchyma in *E. tauvina* spleen was also observed. The mesenchyma was the supporting network consisting of reticular cells, reticular fibres, collagen fibres, arteries, veins, capillaries, etc. While parenchyma was composed of numerous blood sinuses, epithelial cells, lymphoblasts, and blood cells. The facts

observed in the study were in conformity with similar findings in other species (Anderson, 1974; Zapata, 1982; Bly, 1985; Zapata and Cooper, 1990; Quesada *et al.*, 1990; Kennedy-Stoskopf, 1993; Press *et al.*, 1994). The parenchyma consisted of lymphoid tissues carrying several types of lymphoid cells and it was supported by reticular connective tissue. This finding was very much in conformity with similar studies by others (Ellis, 1977; Grace and Manning, 1980; Botham and Manning, 1981; Bly, 1985; Doggett and Harris, 1987; O'Neill, 1989a, b; Razquin *et al.*, 1990). In addition to the above structures, the small capillaries were surrounded by a sheath of reticular connective tissue harbouring numerous macrophages and lymphoid cells. The findings of the study were similar to the studies of others and they designated the sheathed vessels as ellipsoids (Anderson, 1974; Zapata, 1982; Bly, 1985; Zapata and Cooper, 1990; Quesada *et al.*, 1990; Kennedy-Stoskopf, 1993; Press *et al.*, 1994). Several such ellipsoidal blood vessels were noticed in *E. tauvina* spleen. Other workers too reported the presence of ellipsoids in teleosts. Yoffey (1929) indicated that such development of ellipsoids in teleosts was even poorer than that of elasmobranchs. Few pale coloured focal melanomacrophage centers were also observed very near to ellipsoids in *E. tauvina* spleen. This finding was similar to that of plaice (Ferguson, 1976; Zapata, 1983; Zapata and Cooper, 1990).

The special staining reactions of Trichrome-Masson, Verhoeff's-Van Gieson and Reticulum-Gomori revealed important structural details. The spleenic ellipsoids were enclosed within reticular collagen fibre sheath. The reticular collagen fibre sheath around the blood vessels formed a stroma for housing macrophages, lymphocytes and also for antigen trapping. The reticular sheath networks of ellipsoids were in continuity with the melanomacrophage centres. The observations were similar to earlier studies in plaice (Ferguson, 1976; Quesada *et al.*, 1990; Press *et al.*, 1994).

The fine structure of *E. tauvina* spleen was carried out to observe the types of cells present in blood sinuses in detail. The spleen of *E. tauvina* revealed the phenomenon of haemopoiesis in blood sinuses. The blood sinuses were filled with numerous erythrocytes, granulocytes, lymphocytes and macrophages. These cells were very close to endothelial cells present in the capillaries and blood sinuses. The present

finding was similar to the findings reported earlier by others in teleosts (Ferguson, 1976; Zapata, 1982; Quesada *et al.*, 1990; Zapata and Cooper, 1990; Press *et al.*, 1994). Further Quesada *et al.* (1990) reported that there was difference in the reticular cells present in white and red pulp of sea bass (*Dicentrarchus labrax*) spleen. In the present work such differentiation could not be observed.

5.5.4 Gut associated lymphoid tissue (GALT)

In the present study, gut associated lymphoid tissue was well developed and was similar to all other teleosts in structural configuration (Fichtelius *et al.*, 1968; Tomonaga *et al.*, 1986; Hart *et al.*, 1988; Zapata and Cooper, 1990; Zapata *et al.*, 1996). The histomorphological investigation in the present study shows accumulation of lymphocytes in between epithelial cells. Below the epithelial layer and in the loose connective tissue area heavy accumulation of lymphocytes and eosinophilic granule cells were also noticed. The facts observed in the present study are in conformity with studies in many species of teleosts (Zapata *et al.*, 1996; Moore and Hawke, 2004). The observations in the present study have shown the presence of eosinophilic granulocytes and are certainly in agreement with several earlier findings (Zapata *et al.*, 1996; Moore and Hawke, 2004). More detailed studies are needed to confirm the defensive role of GALT in *E. tauvina*.

5. 6 INFLAMMATION

The grouper, *E. tauvina* used in the present study revealed certain inflammatory responses. In the present study, Freund's complete adjuvant was used as experimental inflammation inducing agent. This adjuvant had been used by Finn and Nielsen (1971a, b) in the rainbow trout, *Oncorhynchus mykiss* and later by many authors in several other teleosts. The adjuvant had been popularly used as an inducing agent by many authors for its slow and gradual release of irritants at the site of injection and may therefore give better inflammatory reaction. The Grouper, *E. tauvina* exhibited an acute inflammatory response against Freund's complete adjuvant. Numerous neutrophils, macrophages and lymphocytes were seen in the blood capillaries and were noticed to migrate towards the site of injection. A biphasic leucocyte migration pattern of

neutrophils followed by macrophages was evident. Interestingly many of them were found infiltrating and accumulating at the site of injury. The results are in agreement with the other studies (Roberts, 1989; Suzuki and Iida, 1992). Extensive fibrinous exudate containing neutrophils, lymphocytes and fibrin was also seen at the site of injection. These observations on acute inflammatory responses were similar to the results obtained in other fishes (Finn and Nielsen, 1971a, b; Phromsuthirak, 1977; Sommer and Bartos, 1981; MacArthur *et al.*, 1984; Pulsford and Mattews, 1984; Sovenyi and Baros, 1986; Suzuki and Hibiya, 1986; Park and Wakabayashi, 1989, Hoole, 1994). Some authors claimed the presence of basophils and eosinophils in the inflammatory response. This has not been ascertained in any fish (Suzuki and Iida, 1992; Moore and Hawke, 2004).

5 .7 PHAGOCYTOSIS

Studies on the distribution of injected colloidal carbon particles in the grouper, *E. tauvina* showed points of similarity with other fishes as described by Woodhead (1981) and Manoj (1996). In *in vivo* phagocytosis, colloidal carbon particles are used as stimulus in many fishes (Mackmull and Michels, 1932; Roberts, 1978; MacArthur and Fletcher, 1985; Secombes, 1996). In the present study, Indian ink was used as source of colloidal carbon particles as in earlier studies. Intraperitoneal injection of Indian ink in the species showed the presence of carbon filled phagocytes in the peritoneum after 24 h. The phagocytes were moderate in number and approximately 15 to 20 cells were noticed per high power field. The present result is in conformity with the earlier studies (Manoj, 1996). Further detailed investigations on the species is needed to understand the types of phagocytes involved in phagocytic mechanisms. *In vitro* phagocytic studies of the species using yeast cells revealed phagocytic index of 29 and 32.5 % and the mean was 31.15 ± 0.46. The phagocytic index obtained in the present investigation is less when compared to the earlier reports in tilapia, *Oreochromis mossambicus* (Manoj, 1996).

5.8 CELL MEDIATED IMMUNITY

Plant lectin, phytohaemaglutinin, induces T cell proliferation (Manning and Nakanishi, 1996). This property of phytohaemaglutinin has been used extensively to study T cell response in bony fishes and cartilagenous fishes (Cooper, 1971; Lopez *et al.*, 1974; Sigel *et al.*, 1978; Pettey and McKinney, 1981; Haynes and McKinney, 1991; DeKoning and Kaattari, 1992). In the teleosts T cell and B cell mitogen response has been established (Etlinger *et al.*, 1976; Smith and Braun-Nesje, 1982; Warr and Simon, 1983; Caspi and Avtalion, 1984; Sizemore *et al.*, 1984; Rowley *et al.*, 1988; Tillit *et al.*, 1988; Reitan and Thuvander, 1991).

Assessment of cell mediated immunity (CMI) was carried out in *E. tauvina*. In the present study, phytohaemaglutinin was injected intradermally in the caudal peduncle region to study the delayed hypersensitivity response. There was delayed hypersensitivity response after 24h as evidenced by the increase in skin thickness. The thickness was maintained further up to 72 h. The rate of change in thickness after injection indicated the presence of cell mediated immune response. The present observation established a strong phytohaemaglutinin response and indicated the presence of thymus mediated cells. The observation was in conformity with the earlier studies in channel catfish, common carp and rainbow trout (Warr and Simon, 1983; Sizemore *et al.*, 1984; Caspi and Avtalion, 1984; Tillit *et al.*, 1988; Reitan and Thuvander, 1991) and recently in rohu and pearl sport (George, 1998; Anikuttan, 2004). The present study established that grouper could respond to the antigens that elicit cell mediated immune response.

Further evaluation of peripheral lymphocytes using erythrocyte rosette formation technique (Steward, 2002) yielded approximately 40% rosette forming lymphocytes. This indicated the dichotomy in the population of lymphocytes. In mammals and birds erythrocyte rosette formation with allogenic erythrocytes occurs in the case of lymphocytes with T receptors. Erythrocyte rosette formation has been demonstrated in case of fish with rabbit erythrocytes (Cuchen and Clem, 1977). However, there is no conformation about the lymphocytes of fish forming rosette with erythrocytes of allogenic species.

5. 9 Conclusion

The present study has generated information on the peripheral blood leucocytes and lymphoid organs of *E. tauvina*. It also provided insight into the process of inflammation, phagocytosis and cell mediated immunity.

Based on light and electron microscopy, the leucocytes were classified into neutrophils, eosinophils, lymphocytes and monocytes. Among the granulocytes, two types of granulocytes were identified. The ultrastructure study supported these findings.

Among the lymphoid organs, thymus of *E. tauvina* is divided into several lobules and these lobular structures were supported by reticular connective tissue. Lymphoblasts develop in close proximately with the epithelial cells. The spleen had ellipsoid structures for their blood vessels and lymphopoiesis occurred mostly in the sinuses of the spleen. The kidney performed lymphopoietic as well as excretory functions. Haemopoietic tissue occupied the anterior kidney and intertubular area of trunk and posterior kidney. *E. tauvina* exhibited strong inflammatory responses and responded well to T-cell mitogen-phytohaemaglutinin.

The serum protein study showed 8-10 fractions of these only 4-5 fractions were prominent. The first fraction formed 21.23 ± 1.5%. The mean of second fraction was 19.03 ± 1.59% and third fraction mean was 20.45± 0.71% and the fourth fraction mean was approximately 23.1± 2.01%. In some fishes the fifth fraction was absent whereas majority of fishes had a fifth fraction which formed a mean value of 22.10± o.60%. The first fraction may contain albumin and last fraction may be containing immunoglobulin which formed about more than 20% of the total proteins.

SUMMARY

6. SUMMARY

Grouper aquaculture industry has been severely affected by several infectious diseases, which are contributed by various pathogens. In recent years, several strategies have been applied to control the various economically important diseases. It is well known that a pathogen can cause disease only if it can overcome both nonspecific and specific barriers of the host and successfully establish and proliferate.

The greasy grouper, *Epinephelus tauvina* is one of the major candidate species suitable for culture in saltwater ponds and cages all over the world. Enormous mariculture potential exists for this species in India. The intensive culture practices will definitely pave way for the occurrence of various diseases. Therefore, there is an urgent need to understand the immune system of this species.

In the present study, healthy fishes of body weight 30 - 420 gms were used for various experiments. In the experiment on lymphoid organs, thymus, kidney, spleen and intestine were dissected out for the histology and transmission electron microscopy. Routine histological techniques using haematoxylin and eosin staining along with other special staining methods like Trichrome – Masson, Reticular- Gomori and Verhoeff's Van Gieson were employed during the study. The results are briefly summarized as follows:

- Thymus gland is a well developed, paired organ present on the dorsolateral region of the gill chamber.
- The routine histology revealed that the entire thymus was surrounded by a connective tissue capsule that projected as several trabeculae into the thymic parenchyma. The differentiation of thymic parenchyma into cortex and medulla was not seen.
- Special staining of the thymus revealed that the capsule and trabeculae contained large amount of collagenous tissue. The trabeculae extended

into the parenchyma and it carried the blood vessels and nerves. The connective tissue of the trabeculae also sent collagen tissue into the matrix. In the parenchyma the main supporting tissue of the matrix was formed by reticular fibres. These reticular fibre networks divided the thymus into small lobules. The lobules contained epithelial cells in close association with thymocytes/ lymphoblasts.

- The ultrastructure of the thymus showed many blood capillaries and surrounded by epithelial cells. The capillary lumen was filled with blood cells. Most of the blood capillaries are lined by endothelial cells with tight junctions. Occasionally some of the epithelial cells were fenestrated in appearance while others had electron dense cytoplasm. In association with epithelial cells and in the lumen of capillaries numerous developing lymphoblasts were seen.

- The kidneys of the grouper were paired, capsulated, dark-red organs extending ventrally along the vertebral column and each was separated into anterior and posterior regions.

- The anterior kidney of the species was constituted exclusively of haemopoietic tissues supported by reticular connective tissue while the posterior kidney was formed of nephrons surrounded by haemopoietic tissue and stromal tissue. Numerous blast cells were observed in the stromal tissue of posterior kidney. Numerous blood sinuses lined with endothelial cells were observed in both anterior and posterior kidneys. Developing leucocytes were also observed within and outside the sinuses. Melanomacrophages centers were also found among the haemopoetic tissue.

- The ultrastructure of kidney revealed the presence of numerous lymphoblasts and granulocytes in the cellular matrix. Some of the granulocytes observed were containing crystalloid granules while others

had dense cytoplasmic granules. The parenchymal tissues contained fibroblasts, mesenchymal cells and numerous blood sinuses.

- The spleen was observed very near to the greater curvature of the stomach or flexure of intestine and appeared as a dark red, elongated, cylindrical structure.

- The spleen was composed of both white and red pulps which were not well differentiated. The spleen was surrounded by collagenous splenic capsule composed of fibrous connective tissue. The random distribution of mesenchyma and parenchyma could be seen. The mesenchyma was the supporting network consisting of arteries, veins, capillaries, reticular cells, reticular fibers etc. While parenchyma was composed of numerous blood sinuses and blood cells. The haemopoietic cells, blood vessels and capillaries were found on a mesh work formed by supporting reticular connective tissue. The capillaries were ensheathed in a reticular connective tissue frame work designated as splenic ellipsoids. Few pale coloured focal melanomacrophage centers were also observed very near to ellipsoids.

- The special staining reactions of the spleen revealed that the spleinc ellipsoids were enclosed with reticular and collagen fibre sheath. This reticular collagen fibre sheath around the blood vessels formed the stroma for housing macrophages, lymphocytes and also for antigen trapping. Similar network of reticular sheath was also noticed extending to the melanomacrophage centres.

- Transmission electron microscopy of the spleen revealed the phenomenon of haemopoiesis in blood sinuses. The blood sinuses contained numerous erythrocytes, granulocytes, lymphocytes and macrophages. These cells were very close to the endothelial cells lining the capillaries and blood sinuses.

- Intestinal mucosa was thrown into several projecting folds which were lined by columnar epithelium having a brush boarder appearance at the apical region. The epithelial layer contained numerous secreting goblet cells. There were several lymphocytes occupying the region in between the epithelial cells. Below this, a loose connective tissue area was noticed in which numerous lymphocytes were found scattered. Among the lymphocytes, occasionally cells having eosinophillic granules were found. Further deep, the submucosa was formed of connective tissue containing thick accumulation of lymphocytes and several eosinophilic granular cells.

In the experiment on peripheral blood leucocytes, the leucocytes were characterized by means of differential counts, cytochemical techniques and transmission electron microscopy. The results are briefly summarized as follows:

- The differential counts of leucocytes revealed mainly four types of cells neutrophils, eosinophils, lymphocytes and monocytes.

- The mean percentage of granulocytes was 31.83 ± 6.48. Majority of the granulocytes had unlobed nuclei or indented nuclei while some had lobed nuclei. The cytoplasm contained granules some of which stained mildly basophilic while others took mild eosinophilic stain. The cytochemical studies showed many of these cells were positive for PAS, peroxidase and stained with Sudan black. They were mildly acid phosphatase positive. The ultrastructural studies of granulocytes revealed two types of granules. In one type, numerous uniformly homogenous electron dense granules were abundant in the cytoplasm. In the second type, granules had a peripheral electron dense area and the central portion appeared vacuolated.

- The mean percentage of the monocyte was 6.33 ±1.30. They had a large eccentrically placed nucleus with a foamy or vacuolated cytoplasm. The cytoplasm was mildly positive for PAS and acid phosphatase. The ultrastructural studies revealed cytoplasm with a number of mitochondria,

vacuoles, lysosomes, Golgi apparatus and a few stacks of rough endoplasmic reticulum.

- The mean percentage of lymphocytes was 61.83 ± 2.67. Two types of cells were observed based on the size. In one type, the cells had large nucleus with peripheral cytoplasm. The cytoplasm appeared slightly basophilic and formed a rim around the nucleus. In the other type, the cells appeared small with the nucleus occupying the entire cell. The ultrasructural studies revealed large nucleus with very prominent euchromatin and peripheral heterochromatin. The cytoplasm contained a few mitochondria and some amount of rough endoplasmic reticulum and abundant polyribosomes.

- Thrombocytes were elongated, spindle shaped cells. The ulstrastructural studies revealed the cytoplasm with numerous vacuoles which were interconnected and opened to the exterior through fenestrae.

In the experiment on haematological studies, total erythrocyte and leucocyte counts, total serum protein, albumin, globulin and albumin and globulin ratio were estimated and the results are briefly summarized as follows:

- The mean erythrocyte and leucocyte counts of peripheral blood were 4.42 × $10^6/mm^3$ ± 0.69 and 72.75 × $10^3/mm^3$ ± 0.86 respectively.

- The total leucocyte count showed high degree of correlation ($p < 0.05$) with the body weight.

- The total serum protein values showed wide variation and ranged from 0.16 gm % to 3.32 gm % with mean 1.99 ± 0.24 %.

- The total serum albumin values showed wide variation and ranged from 0.008 gm% to 0.840 gm% with mean 0.14 ± 0.04 %.

- The total serum globulin values showed wide variation and ranged from 0.157 gm % to 3.270 gm % with mean 1.85 ± 0.23 %.

- The A/G ratio showed variation from 0.01 to 0.34 with mean 0.09 ± 0.01. The A/G ratio showed high degree of correlation ($p < 0.05$) with the body weight of fish.

- The serum proteins were separated into 8 -10 fractions. Of these, 4 - 5 fractions were prominent and readable in densitometer. The fractions were representative of various serum proteins and those were 1st fraction (21.23 ± 1.15 %), 2nd fraction (19.03 ± 1.59 %), 3rd fraction (20.45 ± 0.71%), 4th fraction (23.17 ± 2.01%) etc.

- The fast moving fractions may contain albumin followed by various fractions of globulins. The last moving fractions may be containing immunoglobulins.

In the experiment on inflammatory response studies, Freund's complete adjuvant was used to elicit inflammatory response. The results are briefly summaried as follows:

- The response observed was acute inflammatory response.

- Numerous neutrophils, lymphocytes and macrophages were seen in the blood capillaries and were noticed migrating towards the site of injection.

- A biphasic leucocyte migration pattern, neutrophils and lymphocytes followed by macrophages was evident.

- The exudates contained fibrin, neutrophils, lymphocytes and monocytes and these were found accumulated at the site of injection.

- Neither basophils nor eosinophils were noticed at the site of injury.

In the experiment on phagocytosis, *in vivo* and *in vitro* phagocytic responses were studied by using Indian ink and commercial yeast suspension respectively. The results are briefly summarized as follows:

- Intraperitoneal injection of Indian ink after 24 h showed presence of carbon filled phagocytes in the peritoneum.

- The phagocytes were moderate in number and approximately 15 to 20 cells were noticed in a high power microscopic field.

- The leucocytes collected from peripheral blood showed a phagocytic index, which varied between 29 and 32.5 % and the mean percentage was 31.15 ± 0.46.

- It was found that majority of the cells were not phagocytic.

In the experiment on skin sensitivity studies, the T-cell mitogen phytohaemaglutinin (PHA) was injected intradermally at the caudal peduncle of the fishes and skin thickness at the caudal peduncle was measured. The results are briefly summaried as follows:

- There was a significant increase in the thickness of skin that developed after 24 h in caudal peduncle. This was maintained till 72 h.

In experiment on rosette formation, the leucocytes were suspended along with rabbit blood erythrocytes for 12 h at 10^0 C in HBSS and the number of cells forming rosette with erythrocytes were estimated. The results are briefly summarized as follows:

- It was found 39.67 ± 0.71 % of peripheral blood cells formed rosette with rabbit erythrocytes.

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7. REFERENCES

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ANNEXURE-I

COMPOSITION OF ACID PHOSPHATASE SUBSTRATE SOLUTION

1. *Acid phosphatase substrate solution - :30 ml
2. Fast blue BBN :30 mg

***Acid phosphatase substrate solution (100 ml) contains**

(Stable for several months in refrigerator)

1. Naphthol AS Phosphate :30 mg
2. N, N-dimethyl formamide :0.25 ml
3. * *Acetate buffer (at pH 5) :100 ml

****Acetate buffer** (1 M, pH 5)

1. 13.6% Sodium acetate ($CH_3.COONa.\ 3\ H_2O$) :100 ml
2. Acetic acid (6 %) :50 ml

ANNEXURE-II

COMPOSITION OF MYELOPEROXIDASE INCUBATION MIXTURE

1. Ehtyl alcohol (30 %) :100 ml
2. Benzidene dihydrochloride :0.3 gm
3. Zinc sulphate (0.132 M, 3.8 % w/v) :1.0 ml
   ($ZnSO_4$. 7 H_2O)
4. Sodium acetate :1.0 gm
   ($Na_2C_2H_3O_2$. 3 H_2O)
5. Hydrogen peroxide (3%) :0.7 ml
6. 1N Sodium hydroxide :1.5 ml
7. Safranin O :0.2gm

ANNEXURE-III

COMPOSITION OF REDUCING BATH, SCHIFF'S REAGENT AND CELESTINE BLUE SOLUTION

Reducing bath chemical composition

1. Distilled water :100ml
2. Hydrochloric acid (1N) :5ml
3. Sodium bisulphate(10%) :6ml

Celestine blue solution

1. Distilled water :100 ml
2. Ferric ammonium sulphate :5gm
3. Hydrochloric acid :0.5 ml
4. Celestine blue :0.5 gm
   (to be added under 100^0 C)

Schiff's reagent

1. Distilled water :200 ml
2. Basic Fuschin :1.0 gm
3. Normal hydrochloric acid :20.0 ml
4. Sodum metabisulphite :1.0gm
   ($NA_2S_2O_5$)

ANNEXURE-IV

COMPOSITION OF SUDAN BLACK B SOLUTION

Working Sudan black B stock solution

1. *Sudan black B stock solution :60 ml
2. **Buffer solution :40 ml

***Sudan black B stock solution**

1. Sudan black B :0.3 gm
2. Absolute alcohol :100 ml

****Buffer solution**

1. Hydrated disoduim hydrogen phosphate (Na_2HPO_4. 12 H_2O) :0.3 gm
2. Distilled water :100 ml
3. Phenol :16 gm
4. Absolute alcohol :30 ml

ANNEXURE-V

ACETONE GRADIENT USED IN TRANSMISSION ELECTRON MICROSCOPY

1. 30%	:Two changes of 20 min each
2. 50%	: Two changes of 20 min each
3. 70%	:Two changes of 20 min each
4. 80%	:Two changes of 20 min each
5. 85%	:Two changes of 20 min each
6. 90%	:Two changes of 20 min each
7. 95%	:Two changes of 20 min each
8. Absolute acetone	:Three changes of 30 min each

ANNEXURE-VI

ALCOHOL GRADIENT USED IN AUTOMATIC PROCESSOR FOR HISTOLOGY

1. 50%	:2hrs
2. 70%	:2hrs
3. 90%	:2hrs
4. Absolute alcohol	:1hr
5. Propanol-I	:1hr
6. propanol-II	:1hr
7. Propanol-III	:1hr

ANNEXURE-VII

Separating gel (7.5 %):

1. Polyacrylamide stock (30 %) :7.5 ml
2. 1.5 M Tris-Hcl (Ph 8.8) with TEMED* :6.25 ml
3. Double distilled Water :13.0 ml
4. Ammonium per sulphate (10 %) :400 µl.

Total Volume :25 ml

Polyacrylamide stock contained 9.1 % acrylamide and 0.9 % bis-acrylamide. 400 µl Ammonium persulphate was m de upto 25 ml before mixing with the above contents to cast the separating gel.

Stacking gel (3.5 %):

1. Polyacrylamide stock (30 %) :3.0 ml
2. 0.5 M Tris-Hcl (Ph 6.8) :6.25 ml
3. Double distilled Water :15.9 ml
4. Ammonium per sulphate (10 %) :100 µl.
5. TEMED :25 µl

Total Volume :25 ml

Loading buffer:

1. Glycerol :1 ml
2. β-mercapto Ethanol :0.5 ml
3. 0.5 M Tris-Hcl (Ph 6.8) :0.9 ml

TEMED – N, N, N', N'-tetramethylethylenediamine